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Regeneration and inhibition of proton pumping activity of bacteriorhodopsin blue membrane by cationic amine anesthetics

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Abstract

Bacteriorhodopsin (bR) is the prototype of an integral membrane protein with seven membrane-spanning α-helices and serves as a model of the G-protein-coupled drug receptors. This study is aimed at reaching a greater understanding of the role of amine local anesthetic cations on the proton transport in the bR protein, and furthermore, the functional role of "the cation" in the proton pumping mechanism. The effect of the amine anesthetic cations on the proton pump in the bR blue membrane was compared with those by divalent (Ca^{2+}, Mg^{2+} and Mn^{2+}) and monovalent metal cations (Li^{+}, Na^{+}, K^{+} and Cs^{+}), which are essential for the correct functioning of the proton pumping of the bR protein. The results suggest that the interacting site of the divalent cation to the bR membrane may differ from that of the monovalent metal cation. The electric current profile of the bR blue membrane in the presence of the amine anesthetic cations was biphasic, involving the generation and inhibition of the proton pumping activity in a concentration-dependent manner. The extent of the regeneration of the proton pump by the additives increased in the order of monovalent metal cation<monovalent amine anesthetic cation<divalent metal cation. We found that organic cations such as the amine anesthetics can also regenerate the proton pump in the bR protein. The inhibition of proton transport in the bR protein by the anesthetic cations was elucidated using the wild type, the E204Q and the D96N mutated bRs. The hydrophobic interaction of the amine anesthetics with the bR protein plays an important part in inhibiting the bR proton pump.

Keywords: Oriented blue membrane; Cationic amine anesthetic; Proton pump regeneration; Proton pump inhibition; Biphasic effect; Binding site

1. Introduction

Bacteriorhodopsin (bR) serves as a simple model for certain cell-membrane receptors, known as the G-protein-coupled receptors, with include most well-known drug targets in humans [1–3]. bR in purple membrane of *Halobacterium salinarium* functions as a light-driven proton-pump, which uses light energy to translocate protons across the membrane and thereby generates a substantial electrochemical gradient [4–8]. bR in the purple membrane contains seven transmembrane α-helices that enclose an all-*trans*-retinal chromophore linked via a Schiff base to residue Lys216 [9–13]. The transport cycle consists of the interconversions of a sequence of intermediate state, J, K, L, M, N, and O. The retinal chromophore is central to the proton pumping activity of the protein. The first proton transfer step is from the protonated retinal Schiff base to the negatively charged Asp85 located in the extracellular region, and a proton is then released to the extracellular surface from the proton release residues (Glu194 and

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Glu204) [14–18]. The Schiff base is reprotonated by Asp96 located in the cytoplasmic region [19–21].

The functioning of light-adapted bR in the purple membrane as a proton pump requires the presence of divalent cations, Ca^{2+} and Mg^{2+}, which bind at specific locations inside the protein [22–25] and on the bR membrane surface [26,27,38]. The removal of these cations results in the functional transition from the native purple membrane to the blue membrane with inactivation form of proton pump [22,23]. The most important difference between the purple membrane and the blue membrane is the protonated state of negatively charged Asp85, which is an important part of the counterion to protonated Schiff base. The protonated Schiff base of the blue membranes does not deprotonate upon illumination. In addition to the divalent cations, the monovalent cations, such as Na^+ or K^+, can also regenerate the native purple color [22–24]. These metal cations are absolutely necessary for the correct functioning of the proton pumping of the bR protein.

Recently, it is found that the hydrophobic organic cations, such as the quaternary ammonium cations, can regenerate the native purple form [28,29]. This allows us to be interested in studying the action of amine local anesthetics on the proton pump of the bR membrane as a model of the G-protein-coupled receptors in order to elucidate their diverse effects on various membrane processes. Local anesthetics act primarily on excitable neuronal cell membranes and modify the ion translocation at the membrane channels. In addition, these anesthetics are known to bind also to the other excitable cell membrane affecting their function and to generate diverse effects on various membrane processes[30–32]. Since there is a similarity in structure between visual pigment, the rhodopsin and the β-adrenergic or muscarinic receptors with a bundle of seven transmembrane α-helices [33], the transmission of an electric signal generated by rhodopsin in the photoreceptor is interesting in connection with the nerve system.

We have recently reported that the proton pumping activity of the bR in the purple membrane was reversibly inhibited by adding the alkane derivatives (aliphatic amine (RNH_3^+), 1-alcohols (ROH) and aliphatic carboxylates (ROO^-) [34]. From their dose–response curves, the standard free energies per CH_2 for the adsorption of the alkane derivatives to the site of action in the bR membrane were estimated. The proton pumping of the bR purple membrane was mainly suppressed by the hydrophobic interaction with the alkane derivative. The location of the binding site of the RNH_3^+ to the purple membrane was different from those of ROH and ROO^-.

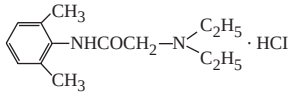
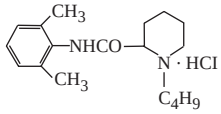
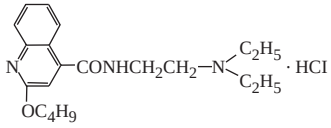
This study is aimed at reaching a greater understanding of the role of the amine anesthetic cations on the proton transport in the bR protein as the G-protein-coupled drug receptors and furthermore, the functional role of "the cation" in the proton pumping mechanism. The electric current profile of the bR protein in the presence of the anesthetic cations was biphasic, involving the generation and the inhibition of the proton pump activity, in a concentration-dependent manner. The results were compared with those by the divalent (Ca^{2+}, Mg^{2+} and Mn^{2+}) and monovalent metal cations (Li^+, Na^+, K^+ and Cs^+), which are essential for the correct functioning of the proton pumping of bR.

2. Materials and methods

2.1. Materials

Purple membrane fragments (ca. 500 nm diameter) were isolated from *H. salinarium* S9. The cation-depleted blue membrane was prepared by passing a purple membrane suspension of OD_{568} ~10 through a cation exchange column (Bio-Rad, AG-50W). The pH of the blue membrane after the column was 5.5–6.0. After deionization, membranes were resuspended in triple-deionized water. The E204Q and D96N mutants were isolated after the expression in *H. salinarium* as purple membrane patches. The tertiary amine anesthetics used were as follows: 2-butoxy-*N*-[2-(diethylamino)ethyl]-4-quinolinecarboxamide (dibucaine · HCl), 4-(butylamino)-benzoic acid 2-(dimethylamino)ethyl ester (tetracaine · HCl), 1-*n*-butyl-2′,6′-dimethyl-2-piperidinecarboxanilide (bupivacaine · HCl), 2-(diethylamino)-*N*-(2,6-dimethylphenyl) acetamide (lidocaine · HCl) and 4-aminobenzoic acid-2-(diethylamine) ethyl ester (procaine · HCl). The structural formulae and the pK_as of the local anesthetics are given in Table 1 [35,36]. Salts ($CaCl_2$, $MgCl_2$, $MnCl_2$, LiCl, NaCl, KCl and CsCl) were guaranteed analytical grade.

Table 1
The structural formula and the pK_a of the tertiary amine anesthetic

Anesthetic	Structural formula	pK_a[35, 36]
Lidocaine (LDC)		7.9
Procaine (PRC)		8.1
Bupivacaine (BPC)		8.1
Tetracaine (TTC)		8.4
Dibucaine (DBC)		8.9

2.2. Preparation of the oriented bR film

The apparatus for the electric measurement was basically the same as that described by Muneyuki et al. [37]. A polymer (polyethylene terephthalate) film (0.9 μm thickness; Lumirror, TORAY CO.) was inserted between the two compartments of the Teflon chamber and the oriented bR blue or purple membrane was prepared by adsorption of its membrane onto the polymer film from the bR membrane suspension; (1) a small amount of the bR membrane suspension (ca. 100 μl, OD_{570}=3 in distilled water) was directly applied on the film sandwiched between the chamber compartments and allowed to stand for 30 min at room temperature. (2) The bR membrane suspension was removed, and pure water (or buffer solution (10 mM Tris/HCl/100 mM KCl, pH 6.0)) was filled in both compartments. (3) Water was exchanged two or three times to wash the unbound bR membrane fragments and then 0.5% (w/v) octylglucoside solution was applied to remove the bR membranes loosely bound on the polymer film and exchanged by the water several times. The adsorbed bR membrane (blue membrane or purple membrane) was equilibrated for 5 min with pure water. A 100 W halogen lamp was used to activate the bR. The intensity of the light at the membrane was 6.6 mW/cm^2. The electric current in bR protein generated by light illumination was measured to evaluate the proton transport activity and recorded before and after exposure to the test substances. Pure water, which was purified by a MilliQ-50 system (Millipore, USA), was used in all this experiment unless otherwise noted in the text.

2.3. Absorption spectra and zeta potential of the purple membrane or the blue membrane suspensions

The absorption spectra were obtained using a JASCO V-500 spectrophotometer (JASCO, Tokyo). The bR blue membranes were dispersed in pure water at 6.2 μM for the absorption spectroscopic measurement. Measurements were carried out at 23±1 °C. The zeta potentials of the purple and blue membranes dispersed in deionized water were measured by the electrophoretic mobility in a Laser-Zee, model 500 apparatus (Pen Kem, New York) at 20±1 °C.

3. Results

The deionization of divalent cations from the bR in the purple membrane is known to result in a purple (λ_{max}=564 nm) to blue color (λ_{max}=603 nm) transition and an inactivation of the proton pump of the bR [22–27]. We confirmed that when divalent cations (Ca^{2+}, Mg^{2+} or Mn^{2+}) were added to the deionized blue membrane suspensions, the spectrum with λ_{max} at 603 nm was blue-shifted in a concentration-dependent manner and then restored to the original purple color with λ_{max} at 564 nm. The addition of the monovalent cations, such as Li^+, Na^+, K^+ and Cs^+, also restored the original purple color (data not shown). These spectral behaviors fairly agreed with previous reports [22,23,26].

Fig. 1 shows the regenerations of the proton pumping activity in the oriented bR blue membrane system by adding the divalent cations (Ca^{2+}, Mg^{2+}) (Panel A) or the monovalent cations (Li^+, Cs^+) (Panel B). The electric current in the bR protein generated by light illumination was evaluated as an indication of the proton pumping activity (see Materials and methods). The relative amplitudes of the electric currents obtained before and after exposure to the metal cation were plotted as a function of the concentration of the additive in pure water. When the divalent cation was added to the deionized bR blue membrane suspensions, the current intensity increased ranging in concentration from 1×10^{-5} to 3 mM and then decreased at concentrations above 3 mM (Fig. 1A), indicative of the biphasic effect of divalent cations on the proton pumping activity in the bR membrane. The addition of the monovalent metal cations also generated electric currents in the bR blue membrane (Fig. 1B) and its amplitude was in the order $Li^+ \leqq Na^+ \leqq K^+ \leqq Cs^+$. The process of the regeneration of the electric current in the presence of the monovalent cation consists of two steps in the ranges of concentration from 1×10^{-5} to 1×10^{-2} mM and from

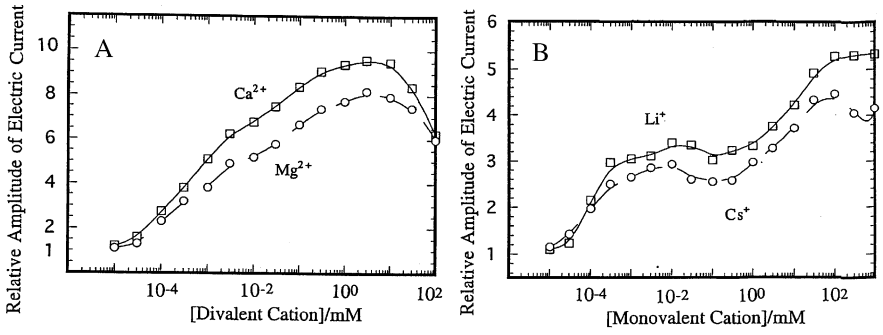


Fig. 1. The regeneration of the proton pumping activity in the bR protein by adding the divalent cation ($Ca2^+$ or Mg^{2+}) (A) or monovalent cation (Li^+ or Cs^+) (B) to the bR blue membrane. The relative amplitudes of the electric currents (pA) in the bR blue membrane obtained before and after exposure to the metal cations are plotted as a function of the concentration of the additive in pure water.

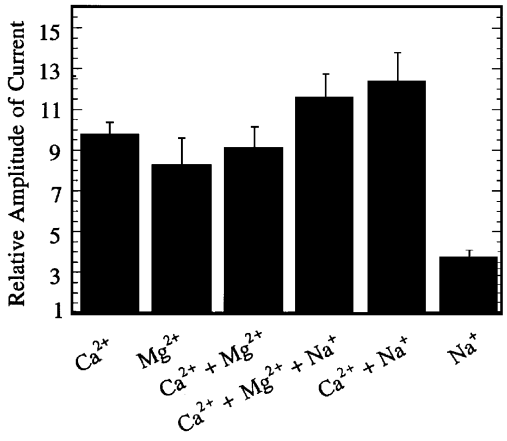


Fig. 2. Comparison of the current amplitude (pA) of the bR blue membrane generated by the addition of a mixture (1/1, molar ratio) of divalent cations (1 mM Ca^{2+} and 1 mM Mg^{2+}), a mixture of three types of cations (1 mM Ca^{2+} and 1 mM Mg^{2+}+1 mM Na^{+}) and a mixture of divalent cation (1 mM Ca^{2+}) and monovalent cation (1 mM Na^{+}) with that by the separate addition of divalent (1 mM Ca^{2+} or 1 mM Mg^{2+}) and monovalent ion (1 mM Na^{+}). The electric current was measured in the presence of metal cation in pure water. The relative amplitude of the electric current is presented as mean values±S.D. (n=3).

2×10^{-1} to 1×10^{3} mM, and the proton pump functions even at concentrations as high as 1 M (Fig. 1B).

In order to examine the location of the interacting sites of the divalent and monovalent metal cations to the bR protein, the current amplitude generated by the addition of a mixture (1/1, molar ratio) of divalent and monovalent cations is compared with that by the separate addition of the divalent or monovalent cations (Fig. 2). The addition of a mixture of divalent cations (1 mM Ca^{2+}+1 mM Mg^{2+}) gave almost the same current amplitude as that of the separate addition of 1 mM Ca^{2+} or 1 mM Mg^{2+}. This suggests that there exist common binding sites of divalent cations which competitively bind with the bR protein. However, the current amplitude generated by the addition of a mixture of the divalent and monovalent cations (1 mM Ca^{2+}+1 mM Na^{+}) or a three component mixture (1 mM Ca^{2+}+1 mM Mg^{2+}+1 mM Na^{+}) is nearly equal to the sum of their values for the separate additions of 1 mM Ca^{2+} and 1 mM Na^{+} or the separate additions of a mixture of the two divalent cations (1 mM Ca^{2+} and 1 mM Mg^{2+}) and of the monovalent cation (1 mM Na+). Therefore, we propose that the locations of the binding sites of the divalent cation and monovalent cation to the bR blue membrane may be different from each other.

Furthermore, we examined the relationship between the electric current and the zeta potential of the bR blue membrane in the presence of various concentrations of Ca^{2+} and Na^{+} (Fig. 3) in order to prove the proposal of different sites for divalent and monovalent ions. The zeta potential of the bR blue membrane was −48.9 mV. The origin of the negative zeta potential is due to the net negatively charged residues in the bR protein and the negatively charged lipid of the bR blue membrane [38]. The divalent cation is much more effective for the generation of the electric current and an increase in the zeta potential of the bR blue membranes than the monovalent metal cation. For example, when 1 mM $CaCl_2$ was added to the bR blue membrane suspensions, the relative amplitude of the electric current and the increments of the zeta potential were 9.4 and 41.4 mV, respectively (Fig. 3A) and the additions of 1 mM NaCl, on the other hand, result in 3.2 in the relative amplitude of the electric current and of 21.4 in the increment of zeta potential, respectively (Fig. 3B). This result also revealed that there may exist different binding sites in the bR blue membrane between the divalent and monovalent cations.

Based on the finding that the hydrophobic organic cations such as $C_{12}H_{28}N^{+}$ or $C_{15}H_{36}N_2^{+}$ can also restore the purple color and regenerate the proton pumping activity in the deionized bR protein [28,29], our attention became focused on the effect of the cationic amine derivatives such as amine local anesthetics (dibucaine, tetracaine, bupivacaine, lidocaine and procaine (Table 1)) on the proton transport in the bR as a model of G-protein-coupled drug receptors. Fig. 4 shows the typical electric current traces in the bR blue membrane generated by adding the tetracaine cation. The traces reveal that the anesthetic cation regenerated the bR proton pump in a concentration-dependent manner. Fig. 5 shows the profiles of the electric currents in the bR blue membrane (Panel A) generated by the addition of five types of amine anesthetic cations and the absorption spectra (Panel B) of the bR membrane after the addition of the tetracaine cation in distilled water (pH 6.0). As the

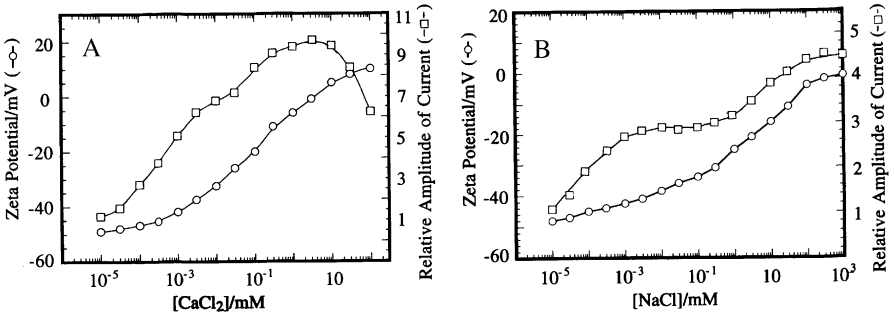


Fig. 3. Relationship between the generated electric current and the zeta potential of the bR blue membrane in the presence of various concentrations of divalent cation Ca^{2+} (A) and monovalent cation Na^{+}(B) ions. Measurements were carried out in the presence of the additives in pure water. The relative amplitude of the electric current is presented as mean values±S.D. (n=3). Circle and square indicate the zeta potential and the electric current, respectively.

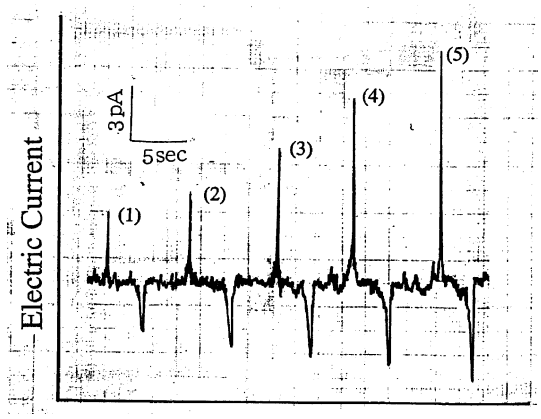


Fig. 4. Electric current traces of the bR blue membrane generated by adding the cationic amine tetracaine. Concentration of tetracaine: (1) control; (2) 0.1; (3) 0.2; (4) 0.7; (5) 3 μM. Upward and downward signals indicate "on" and "off" of the light source, respectively.

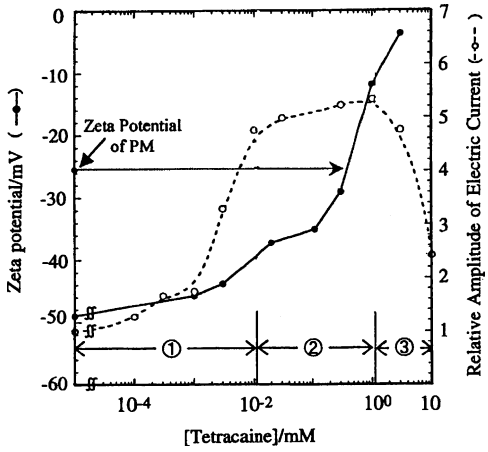


Fig. 6. Relationship between the generated electric current and the zeta potential of the bR blue membrane as a function of the tetracaine concentration in pure water. When the 0.33 mM tetracaine is added to the bR blue membrane, the zeta potential of the bR blue membrane is equal to that (−25.5 mV) of the bR purple membrane (PM) as indicated by the arrow. The dose–response curve of tetracaine is divided into three regions indicated by the solid lines (see text). Solid and dotted lines indicate the zeta potential and the electric current, respectively.

concentrations of the anesthetic cations increased, the electric currents increased, leading to a maximum value, and then decreased (Fig. 5A). The electric current profile in the presence of the anesthetic cation was biphasic, involving the generation and inhibition in the proton pump, in a concentration-dependent manner. Their dose–response curves were explicitly dependent on the type of amine anesthetics. The absorption spectra of the bR membrane in the presence of the tetracaine cation at various concentrations have a single isosbestic point at 580 nm, indicating that only two species are involved in the blue (λ_{max}=603 nm) to purple (λ_{max}=568 nm) transition (Fig. 5B), analogous to the color transition from the blue membrane in the presence of metal cations [22]. The concentration of the monovalent tetracaine cation, which needs to cause the blue to purple transition, is approximately 30 μM, and therefore, the molar ratio of the tetracaine cation to the blue membrane (6.2 μM) is approximately 5:1 (Fig. 5B). This ratio is compared with the molar ratio of approximately 100:1 of the monovalent metal cations, such as Na^+ or K^+, which need to restore the purple color [22]. We found that the regeneration of the native purple color is much more effective for the monovalent organic cations, such as the amine anesthetics, than that for the monovalent metal cation.

Next, we studied the regeneration process of the electric current by the interaction of the anesthetic cation with the blue membrane. Fig. 6 shows the relationship between the electric current and the zeta potential of the bR blue membrane as a function of the cationic tetracaine concentration in pure water. The zeta potentials of the bR blue membrane and the purple membrane were −48.9 and −25.5 mV, respectively. The removal of the metal cations such as Ca^{2+} and Na^+ from the bR purple membrane results in a reduction of the zeta potential. The addition of the 0.33 mM tetracaine cation transformed the blue membrane with a negative potential of −48.9 mV into the purple membrane (−25.5 mV) as indicated by the arrow. The electric current profile in Fig. 6 may be divided into three regions: (1) the

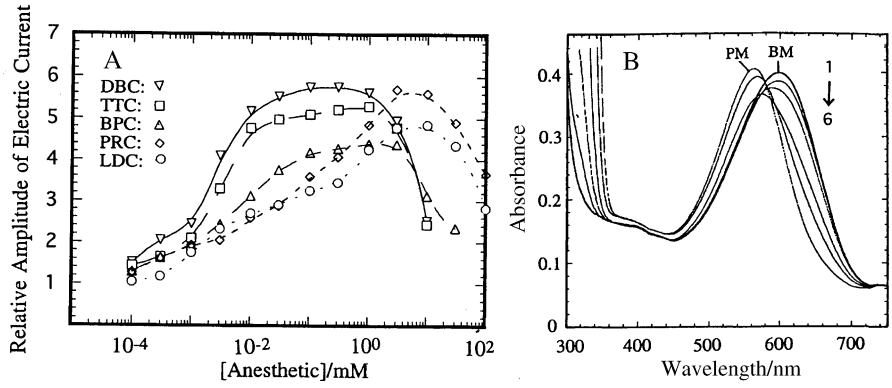


Fig. 5. The effect of amine local anesthetic cations on the bR proton pump (A) and the blue (BM) to purple (PM) color transition (B) by the addition of the tetracaine cation in distilled water. DBC, dibucaine; TTC, tetracaine; BPC, bupivacaine; LDC, lidocaine; PRC, procaine. Concentration of TTC in Fig. 4B: (1) control; (2) 3×10^{-3}; (3) 1×10^{-2}; (4) 0.03; (5) 0.1; (6) 0.3 mM. The addition of 0.3 mM TTC caused the BM (λ_{max}=603 nm) to PM (λ_{max}=564 nm) color transition.

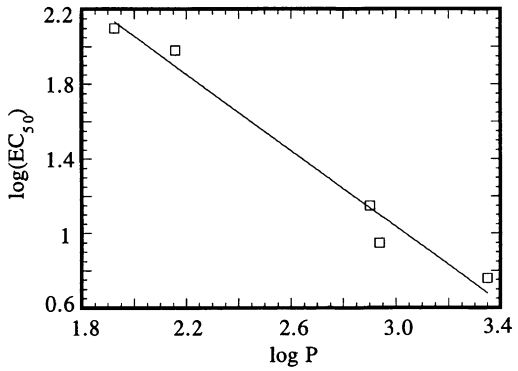


Fig. 7. The relationship between the EC_{50} value and the partition coefficient (P) of local anesthetics between phosphatidylcholine bilayer membrane and water phases. The EC_{50} is defined as the concentration of the local anesthetics required to reduce the amplitude of the electric current of bR to 50% of its initial value. log P: 1.94 for LDC; 2.15 for PRC; 2.89 for BPC; 2.95 for TTC; 3.35 for DBC.

process of the generation of an electric current at concentrations below 0.01 mM tetracaine, (2) the plateau region in the current profile at concentrations from 0.01 to 1 mM, and (3) the process of the reduction in the electric current at concentrations above 1 mM. In region (1), the addition of small amounts of tetracaine significantly increased the electric current in the bR until a maximum current is attained, but the change in the zeta potential is small. In region (2), the maximum current is reached, while the zeta potentials significantly increases with the increasing tetracaine concentration. In region (3), the reduction of the current starts, but the zeta potential still keeps increasing. Although the zeta potentials of the bR membrane in the presence of the tetracaine cation continues to increase in a concentration-dependent manner, the profile of the current generated by the addition of the tetracaine cation is biphasic, including the increasing (generation) and decreasing (inhibition) of the proton pumping activity.

In order to elucidate the inhibition of the proton pump in the region of high concentrations of the anesthetics, the relationship between the EC_{50} value and partition coefficient [39] of local anesthetics between the phosphatidylcholine bilayer membrane and aqueous phases is plotted in Fig. 7. The concentrations of the local anesthetics (EC_{50}), which

Table 2
The effective concentration, EC_{50}, of the cationic tetracaine and Ca^{2+} required to inhibit the proton pumps of Wild Type bR, D96N and E204Q mutants

bR	Tetracaine (mM)	$CaCl_2$ (mM)
WTbR	6.1	290
E204Q	3.1	470
D96N	1.0	23

are required to reduce the amplitude of the electric current to 50% of its initial value, were determined from the dose–response curves in Fig. 5. The obtained linear relationship suggests that the hydrophobicity of the amine anesthetics (Table 1) is closely associated with the inhibition of the proton pumping activity in the bR blue membrane.

Furthermore, we consider the molecular mechanism of the inhibition of the proton pumping activity by the amine anesthetics using the wild-type bR and the D96N- and the E204Q-bR mutants, which are obtained from the point-mutation of key residues involved in the proton transport in the bR protein. Fig. 8 shows the effects of the tetracaine cation (Panel A) and Ca^{2+} (Panel B) on the proton pumping activity in the wild-type and the D96N- and the E204Q-mutated bRs. The measured currents in the absence of the additives were 40–45 pA for the wild type bR, 7 pA for D96N, and 3 pA for E204Q, respectively. The relative amplitude of the electric currents of their mutants to the wild type bR were 16% for the D96N mutant and 7% for the E204Q mutant. All their proton pumps were inhibited by adding the tetracaine cation or Ca^{2+} in a concentration-dependent manner. The electric currents of the wild type bR decreased at concentrations above 0.3 mM tetracaine via the small generation of the proton pump activity ranging in concentration from 0.001 to 0.02 mM, while the electric current of the D96N mutant started decreasing even at the low concentration of 0.03 mM. However, although the pattern of the reduction in the current intensity of the E204Q mutant at high additive concentrations appears to be similar to that of the wild type bR, the electric current of the E204Q may involve large errors (Fig. 8). The effective concentrations (EC_{50}) of the tetracaine cation and $CaCl_2$ required for 50% of the proton pump inhibitions of the wild type and

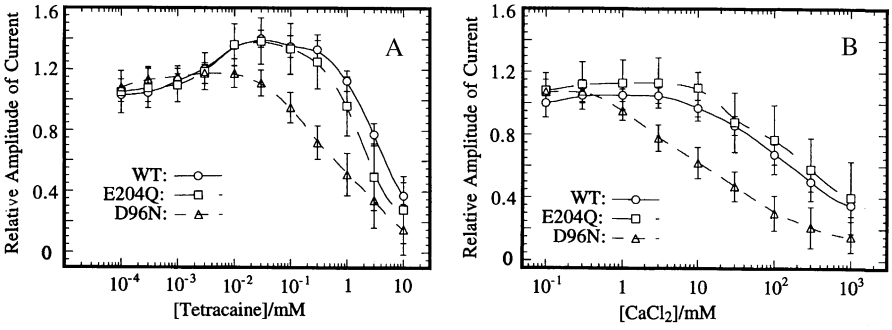


Fig. 8. The effect of the cationic tetracaine (A) and Ca^{2+}(B) on the proton pumping activity of the wild-type (WT), the D96N- and E204Q-mutated bR proteins in 10 mM Tris/HCl/100 mM KCl buffer solution (pH 6.0). The relative amplitude of the electric current is presented as mean values±S.D. (n=3).

the D96N- and E204Q-bR mutated bRs were estimated from the dose–response curves and are listed in Table 2. The EC_{50}s for the tetracaine cation were lower for these mutants than that for the wild type bR.

4. Discussion

We verified that the divalent and monovalent cations were able to regenerate the blue to purple color transition as previously reported [22–25]. Taking into account the data that the bR color transition is coupled to its proton pump [22,23], the effects of the divalent and monovalent cations on the proton pumping activity in the bR blue membranes were examined in the same concentration region as that used in the bR color transition measurement (Fig. 1). When the divalent cations were added to the deionized bR blue membrane, the current intensity of the bR increased ranging in concentration from 1×10^{-5} to 3 mM and then decreased in concentrations above 3 mM (Fig. 1A). This biphasic behavior of the electric currents indicates that there exist at least two types of locations of the binding sites of the divalent cation on the bR blue membrane. The specific sites inside the bR protein [22–25] and the bR membrane surface [26,27,44] are candidates for the location of the binding sites of the divalent cations. Monovalent cations can also regenerate the electric current of the bR blue membrane and their abilities are in the order $Li^+ \leqq Na^+ \leqq K^+ \leqq Cs^+$ (Fig. 1B). This order agrees with Hofmeister series as a measure of the hydration ability of the monovalent metal cations [40]. The hydration ability of these cations as a water structure maker (Li^+, Na^+) or water structure breaker (Cs^+, K^+) is closely associated with the effects of the monovalent metal cations on the blue to purple color transition and the regeneration of the electric current in the bR [40,41].

The current amplitudes generated by the addition of a mixture (1/1, molar ratio) of di- and monovalent cations were compared with those by the separate addition of the divalent or monovalent cations (Fig. 2), in order to examine the location of the interacting sites of the divalent and monovalent metal cations to the bR blue membranes. The results suggest that the divalent cations, Ca^{2+} and Mg^{2+}, have common interacting sites, in which the divalent cations competitively interact with a specific site of the bR protein [42–44]. By comparing the current amplitudes of the bR generated by the addition of a mixture of the divalent cation and monovalent cation (1 mM Ca^{2+}+1 mM Na^+) and the addition of a three component mixture (1 mM Ca^{2+}+1 mM Mg^{2+}+1 mM Na^+) (Fig. 2), we found that the interacting moiety of the monovalent cation to the bR blue membrane may differ from those of the divalent cation [38]. Furthermore, this proposal may be supported by the results in Fig. 3, in which the zeta potential profiles of the bR blue membranes in the presence of the divalent (Ca^{2+}) or monovalent cations (Na^+) significantly differ from each other. When the divalent (Ca^{2+}) or monovalent cations (Na^+) with the same concentration (1 mM) were added to the bR blue membrane suspensions, both changes in the current amplitude and the zeta potential are much greater for Ca^{2+} than those for Na^+.

In addition to the divalent and monovalent metal cations, it has been found that hydrophobic organic cations such as $C_{12}H_{28}N^+$ or $C_{15}H_{36}N_2^+$ can also regenerate the proton pumping activity in the bR blue membrane [28,29]. These reports allowed us to study the role of the amine anesthetics cations (dibucaine, tetracaine, bupivacaine, lidocaine and procaine) on the proton transport of the bR protein as a model of the G-protein-coupled drug receptors [1–3]. The results showed that as the concentrations of the anesthetic cations increased, the currents in the bR blue membranes increased, leading to a maximum value, and then changed to decreasing, indicative of the biphasic effect of amine anesthetics on the bR proton pump (Fig. 1A). This biphasic behavior of the electric currents indicates that there exist at least two types of locations for the binding sites of the anesthetic cation for the current generation and inhibition. Their dose–response curves were explicitly dependent on the type of amine anesthetics (Fig. 5A). The molar ratio of the tetracaine cation to the blue membrane, which causes the blue to purple transition, was approximately 5:1 (Fig. 5B). This ratio was compared with the molar ratio, 100:1, of the monovalent metal cations, such as Na^+ or K^+ [22]. It is noteworthy that the anesthetic cations are much more effective for the regeneration of the native purple color than the monovalent metal cations.

In order to elucidate the interacting site of the anesthetic cation to the bR membrane, we examined the relationship between the changes in the electric current and in the zeta potential of the bR blue membrane by adding the tetracaine cation (Fig. 6). The electric current in the bR protein in the presence of the tetracaine cation was generated by an electrostatic interaction with the negatively charged bR membranes [38]. The obtained current profile is biphasic, involving an increase and decrease in the electric current of the bR in a concentration-dependent manner, while the zeta potential continues to increase even at high concentrations of the tetracaine cation: (1) At the low concentrations below 0.01 mM, the electric current remarkably increased, though the increases in the zeta potential by the tetracaine binding were small. This suggests the existence of specific binding sites of the amine anesthetic cations to the bR [29], including a site near Asp85 within the protein [42,43], or more external sites [44]. (2) At high concentrations above 1 mM, while the zeta potential of the bR blue membrane shows a significant increase, the electric current decreases, and therefore, the proton pump is inhibited. The inhibition of the bR proton pump in the high concentration region of the tetracaine cation is considered to arise from the global conformational change in the bR protein by a nonspecific adsorption of the tetracaine cation within the electric double layer [38] of the negatively charged bR blue membrane surface. (3) At concentrations from 0.01 to 1 mM there

exists a plateau region in the current profile. This plateau may occur by the competition between the regeneration (current increase) and inhibition (current reduction) of the proton pumping activity.

Next, we discuss the mechanism of the inhibition of the proton pumping activity by the amine anesthetics using the wild-type bR and the D96N- and the E204Q-bR mutants, which are obtained from the point-mutation of key residues involved in the bR proton transport (Fig. 8). In the case of the wild type bR, the observed electric current is the sum of the proton transfer from the Schiff base to D85 and from D96 to the deprotonated Schiff base, whereas in the case of the D96N mutant, there is no proton acceptor on the cytoplasmic side and all the protons released from the Schiff base move to D85. The inhibition of the proton pump by the hydrophobic tetracaine cation is much more effective than that by the divalent Ca^{2+} (Table 2), suggesting that the hydrophobic interaction with the bR membrane also plays an important part in inhibiting the proton pump (Fig. 7). The EC_{50} for the proton pump inhibitions in the presence of the tetracaine cation was in the order D96N-bR<wild type bR, and the inhibition was the most effective for the D96N mutant (Table 2). This suggests that the area around the D96 residue, which is located in the hydrophobic cytoplasmic half of the bR molecule, is a candidate for the location of the interacting sites of the tetracaine cation. The first photocycle intermediate is dramatically slowed down when the proton donor D96 in the cytoplasmic halfchannel is replaced with asparagine. In this D96N mutant, the Schiff base eventually reprotonates from the distant cytoplasmic surface for lack of a nearby proton donor [45,46]. It is widely accepted that the bR protein undergoes global conformational changes in the bR during proton pumping and its conformational changes occur in the hydrophobic cytoplasmic half of the bR molecule [46]. Therefore, the interaction of the tetracaine cation with the bR membrane may modify the global conformational changes which occur in the hydrophobic cytoplasmic half of the bR molecule, in which the D96 residue locates [47]. As a result, the reprotonation of the Schiff base from the distant cytoplasmic surface may be interrupted. Although the electric currents of the E204Q may involve a significant error (Fig. 8), their currents tend to decrease in the high concentration regions of the additive. The E to Q point mutation of the E204 residue in the extracellular side may contribute to weakening the hydrogen-bonded network formed through the C=N part of the Schiff base [20,21]. The interaction of the anesthetic cation with the bR membrane affects the hydrogen-bonded network of the residues in the extracellular channel and therefore, pumps proton with difficulty. We found that the amine anesthetic cations significantly suppressed the proton uptake in the cytoplasmic half and the proton release in the extracellular half of the protein by the global conformational perturbation of the bR protein.

5. Conclusion

We examined the effect of the amine anesthetic cations on the proton transport of the bR as G-protein-coupled drug receptors. The obtained results were compared with those of the divalent (Ca^{2+}, Mg^{2+}, Mn^{2+}) and monovalent metal cations (Li^{+}, Na^{+}, K^{+} and Cs^{+}), which are essential for the correct functioning of the proton pumping of the bR. The binding site of the divalent metal cation to the bR protein may differ from that of the monovalent metal cations. The regeneration of the proton pump is much more effective for the monovalent anesthetic cations than those for the monovalent metal cations. The electric current profiles in the presence of the anesthetic cations are biphasic, involving the generation and inhibition of the proton pump, in a concentration-dependent manner. The results provided useful information on the elucidation of the diverse effect of amine anesthetics on various membrane processes, and furthermore, on the functional role of "the cations" in the bR proton pumping mechanism.

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