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Reinvestigation of Calixarene-Based Artificial-Signaling Acetylcholine Receptors Useful in Neutral Aqueous (Water/Methanol) Solution

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Abstract: It was shown that an artificial acetylcholine (neurotransmitter) detection system using resorcin[4]arene (**1**: Inouye et al. *J. Am. Chem. Soc.* **1994**, *116*, 5517) has substantial problems: both acetylcholine and a fluorescent receptor molecule decompose in 0.01 M KOH/MeOH solution. In their system the strong alkaline medium was necessitated to dissociate OH groups in **1** and to enhance the fluorescence quenching ability. To solve this problem we exploited a new artificial acetylcholine detection system using water-soluble calix[*n*]arene-*p*-sulfonates which are useful even in *aqueous (water/methanol) neutral solution.* This achievement is due to several outstanding properties of these calix[*n*]arenes such as low pK_a values, cation−π interactions, high water-solubility, etc.

Introduction

Cation−π interactions have been of much concern as a novel secondary force working in molecular recognition,[1−4] enzyme active sites,[5] ion channels,[6] etc. Calix[4]arene derivatives can provide an ideal architecture for evaluating the contribution of the cation−π interaction in artificial inclusion systems because they possess a π-basic cavity composed of four benzene rings and the various cavity shapes can be easily created by immobilization of the conformational isomers.[7−9] Recently, Inouye et al.[10] designed an elegant artificial-signaling acetylcholine receptor system in which the binding process of cationic guests to the π-basic cavity of resorcin[4]arene (**1**) can be conveniently read out by a fluorescence intensity change (Scheme 1): that is, the fluorescence of a cationic reporter molecule (**2**) is quenched pseudo-intramolecularly when it is bound to tetraanionic host $\mathbf{1}^{4-}$ whereas it gives strong fluorescence when it is replaced with cationic guests. To dissociate four OH groups in **1** they used 0.01 M KOH/MeOH. Previously, we were interested in NAD^+/NADH model systems and knew that pyridinium nuclei as in **2** are very susceptible to nucleophiles.[11,12] They readily react with nucleophiles and yield 1,2- or 1,4-dihydropyridine adducts.[11,12] We carefully inspected the time dependence of the absorption spectra of **2** under the same measurement conditions as those employed by Inouye et al.[10] As expected, the spectral change which is attributable to the formation of the 1,2- or 1,4-dihydropyridine adduct was observed (Figure 1). Furthermore, HPLC analysis showed that acetylcholine in 0.01 M KOH/MeOH undergoes the base-catalyzed methanolysis at room temperature (the sample solution was neutralized with HCl before measurement: two peaks assignable to acetylcholine and methyl acetate appeared in this order). These results reveal that Inouye's system using alkaline conditions is not recommended for the time-dependent monitoring of acetylcholine. Furthermore, it is favorable from a practical viewpoint that this monitoring is carried out in *neutral aqueous solutions.*

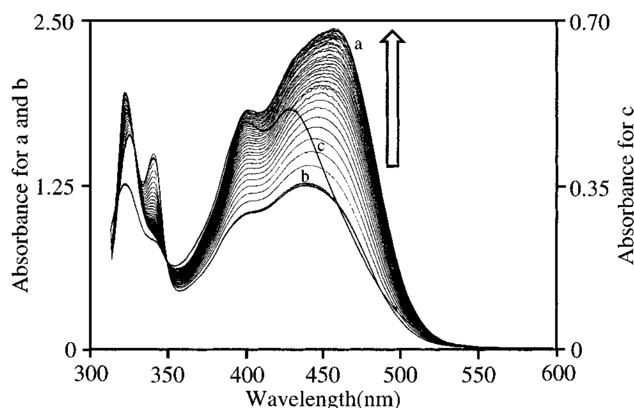

Figure 1. (a) Time dependence of the absorption spectra of **2** (I^- salt: 1.00 × 10^{-4} M) in 0.01 M KOH/MeOH at 25 °C. The time interval for each scan is 60 min. (b) In MeOH. (c) In water (Cl^- salt, the concentration of **2** is 3.33 × 10^{-5} M).

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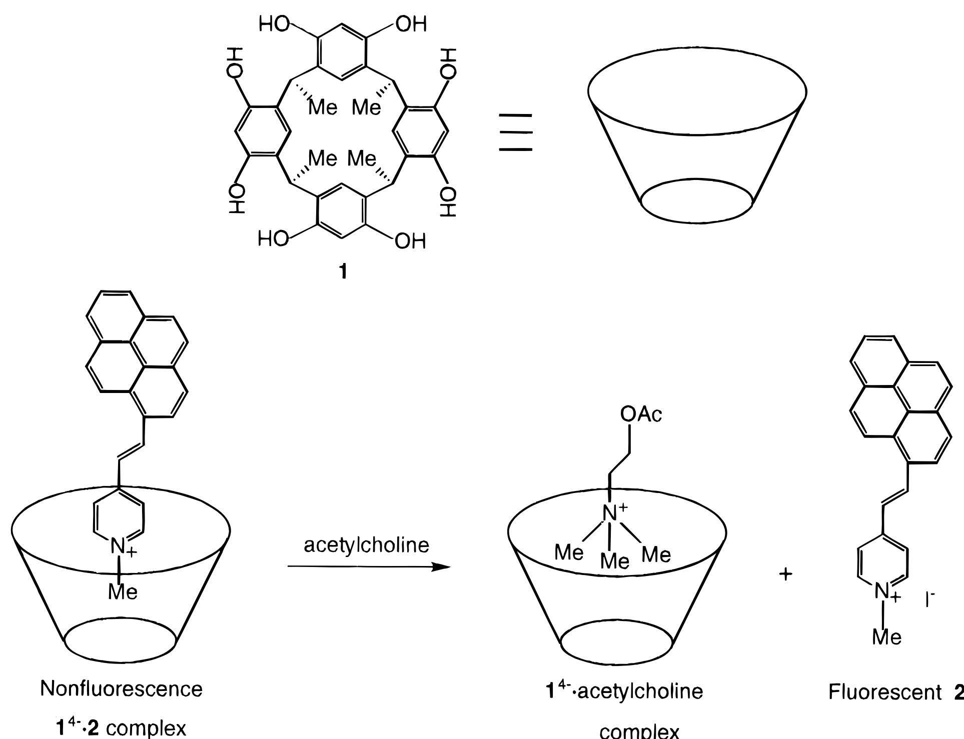
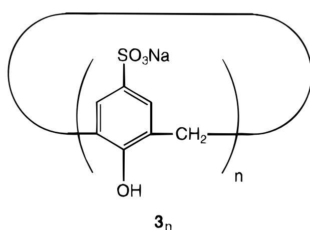
Scheme 1



Then, how can we redesign such an elegant nondestructive acetylcholine detection system? It is known that calix[n]arene-p-sulfonates ($\mathbf{3}_4$ for $n = 4$ and $\mathbf{3}_6$ for $n = 6$) have very low pK_a values[13−15] and can include cationic guests with the aid of cation−π interactions.[3a,16] This implies that compound $\mathbf{3}_n$ may be useful instead of **1** in the neutral pH region where neither the unfavorable adduct formation with **2** nor the methanolysis of acetylcholine takes place. To realize this working hypothesis we here evaluated the function of $\mathbf{3}_n$ as an artificial-signaling acetylcholine (neurotransmitter) receptor.



Results and Discussion

Since the recognition events related to neurotransmitters proceed under physiological conditions, we here employed an 1:1 v/v water/MeOH medium at pH 8.0 (buffered with 0.1 M phosphate: corrected according to Bates' method[17]). The use of MeOH was inevitable in order to suppress the aggregation of **2** in an aqueous system. It was confirmed that under these measurement conditions neither decomposition of **2** nor methanolysis of acetylcholine takes place. Figure 2 shows the

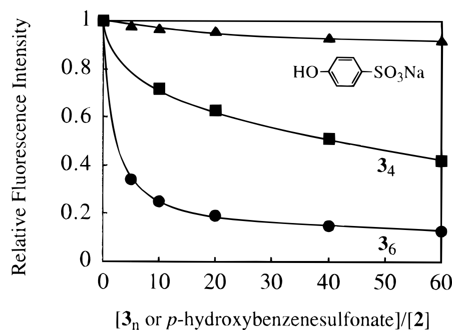


Figure 2. Relative fluorescence intensity of **2** plotted against $\mathbf{3}_n$ or p-hydroxybenzenesulfonate concentration: [**2**] = 1.00×10^{-4} M, 25 °C, water/MeOH = 1:1 v/v, pH 8.0 with 0.1 M phosphate buffer, excitation (isosbestic wavelength in the absorption spectra) 430 nm, emission 580 nm.

fluorescence intensity of **2** plotted against $\mathbf{3}_n$ or p-hydroxybenzenesulfonate (noncyclic reference compound) concentration. It is seen from Figure 2 that the relative fluorescence intensity (I/I_o) is efficiently quenched by $\mathbf{3}_4$ and $\mathbf{3}_6$ according to a typical saturation curve whereas p-hydroxybenzenesulfonate scarcely changes the fluorescence intensity.[18] Judging from the pK_a values of $\mathbf{3}_4$ and $\mathbf{3}_6$ (pK_{a1} = 3.3, pK_{a2} = 12.3, pK_{a3} = 12.9, pK_{a4} = 13.6 and pK_{a1} = 3.5, pK_{a2} = 5.0, p$K_{an,n\geq3}$ > 11.0 at 25 °C, respectively),[13−15] one OH group is dissociated in $\mathbf{3}_4$ and two OH groups are dissociated in $\mathbf{3}_6$ at pH 8.0. The results show that these cavities containing one or two anionic phenolate units can include **2** owing to the cation−π interaction[3a,16] and quench the singlet state of **2** in a pseudo-intramolecular manner. From the analysis of the I/I_o vs $\mathbf{3}_n$ concentration plots by the Benesi−Hildebrand equation[19] the association constants (K_{ass}) were estimated to be $10^{2.84\pm0.03}$ M^{-1} for $\mathbf{3}_4$ and $10^{3.76\pm0.01}$ M^{-1} for $\mathbf{3}_6$ (correlation coefficient >0.99).

The foregoing results establish that $\mathbf{3}_n$ can satisfy the first requirement (i.e., fluorescence quenching of included **2**). The second requirement is related to the fluorescence regeneration which must be induced by selective substitution of **2** with acetylcholine. We tested this requirement using $\mathbf{3}_6$ which gave

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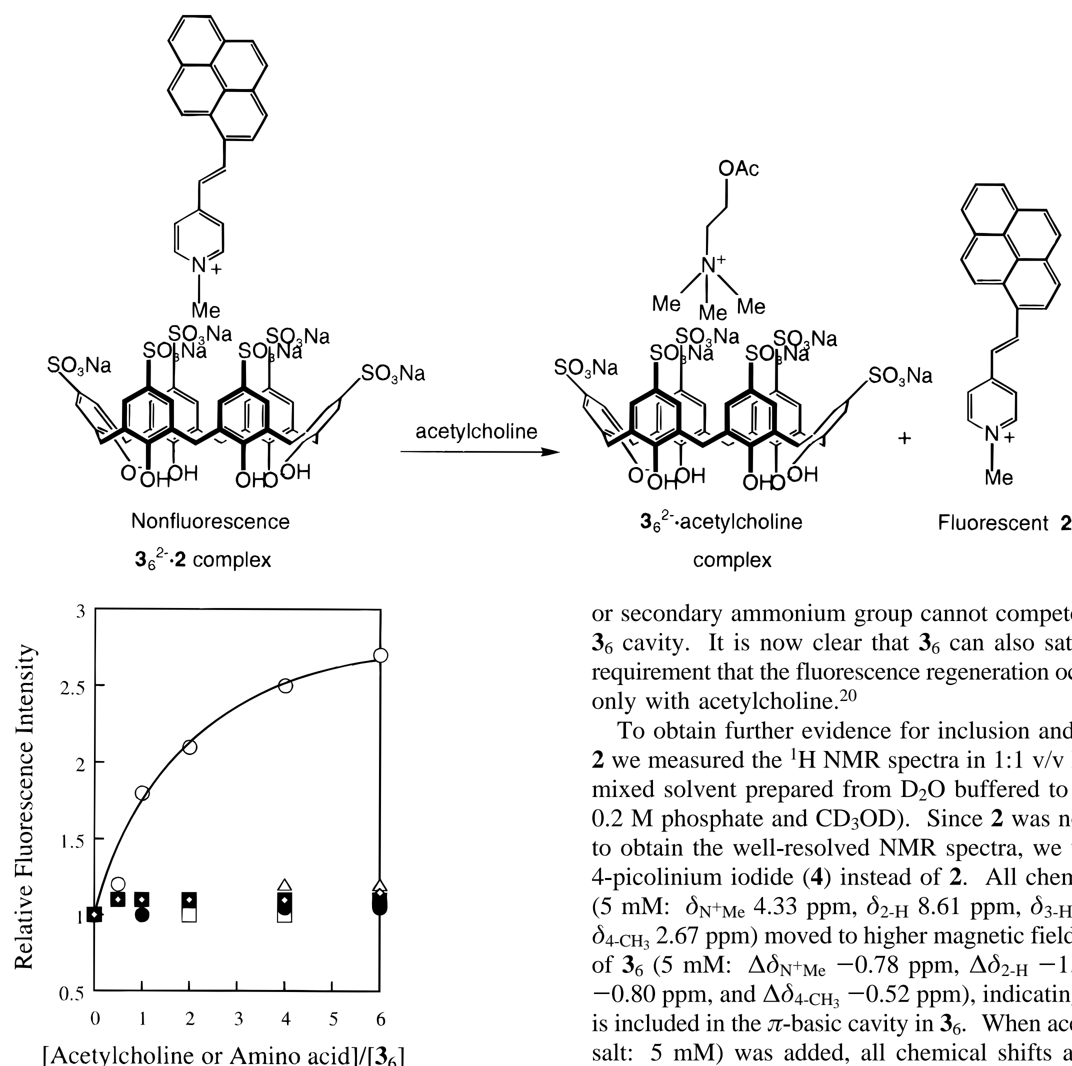
Scheme 2



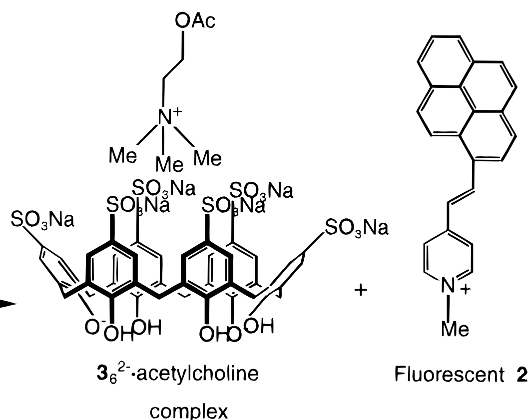


Figure 3. Fluorescence regeneration: [**2**] = 1.00 × 10^{-4} M, [$\mathbf{3}_6$] = 1.00 × 10^{-3} M, (○) acetylcholine, (●) glycine, (△) L-aspartic acid, (□) L-proline, (■) L-phenylalanine ethyl ester hydrochloride, (◇) glycine methyl ester hydrochloride. Other conditions are the same as those in Figure 2.

the larger K_{ass} for **2**. In Figure 3 acetylcholine (Cl^- salt) was added to a 1:1 v/v water/MeOH solution containing $\mathbf{3}_6$ (1.00 × 10^{-3} M) and **2** (1.00 × 10^{-4} M). The fluorescence intensity of **2** increased with increasing acetylcholine concentration, indicating that acetylcholine can substitute **2** bound to the $\mathbf{3}_6$ cavity. From the analysis of the I/I_o vs acetylcholine concentration plots in Figure 3 by the substitution method taking the mass balance into consideration the association constant (K_{ass}) was calculated to be $10^{3.07\pm0.01}$ M^{-1}. In contrast, the increase in the fluorescence intensity was scarcely induced by the addition of amino acids (such as glycine, L-aspartic acid, L-proline, L-phenylalanine ethyl ester hydrochloride, glycine methyl ester hydrochloride, etc.). The results establish that amino acids bearing a primary or secondary ammonium group cannot compete with **2** for the $\mathbf{3}_6$ cavity. It is now clear that $\mathbf{3}_6$ can also satisfy the second requirement that the fluorescence regeneration occurs selectively only with acetylcholine.[20]

To obtain further evidence for inclusion and substitution of **2** we measured the ^{1}H NMR spectra in 1:1 v/v D_2O/CD_3OD (a mixed solvent prepared from D_2O buffered to pD = 7.4 with 0.2 M phosphate and CD_3OD). Since **2** was not so soluble as to obtain the well-resolved NMR spectra, we used *N*-methyl-4-picolinium iodide (**4**) instead of **2**. All chemical shifts of **4** (5 mM: δ_{N^+Me} 4.33 ppm, $\delta_{2\text{-H}}$ 8.61 ppm, $\delta_{3\text{-H}}$ 7.90 ppm, and $\delta_{4\text{-CH}_3}$ 2.67 ppm) moved to higher magnetic field in the presence of $\mathbf{3}_6$ (5 mM: $\Delta\delta_{N^+Me}$ −0.78 ppm, $\Delta\delta_{2\text{-H}}$ −1.12 ppm, $\Delta\delta_{3\text{-H}}$ −0.80 ppm, and $\Delta\delta_{4\text{-CH}_3}$ −0.52 ppm), indicating that this guest is included in the π-basic cavity in $\mathbf{3}_6$. When acetylcholine (Cl^- salt: 5 mM) was added, all chemical shifts attributable to **4** moved to lower magnetic field ($\Delta\delta_{N^+Me}$ 0.30 ppm, $\Delta\delta_{2\text{-H}}$ 0.39 ppm, $\Delta\delta_{3\text{-H}}$ 0.17 ppm, and $\Delta\delta_{4\text{-CH}_3}$ 0.1 ppm from those in the presence of $\mathbf{3}_6$ and **4**) whereas the N^+Me protons in acetylcholine shifted to higher magnetic field (from 3.22 to 2.31 ppm).[21] The results are consistent with the conclusion derived from the fluorescence spectroscopic studies. Based on the foregoing findings, one can now propose Scheme 2 which unequivocally substantiates an artificial-signaling detection system for acetylcholine in an aqueous (water/methanol) system.

Conclusion

The present study offers an essentially improved fluorescent sensing system which is useful for the time-dependent monitoring of acetylcholine in *neutral aqueous (water/methanol) solution*. The success in the molecular design is due to several outstanding characteristics inherent in $\mathbf{3}_n$: that is, (i) the pK_a values of the OH group (first dissociation for $\mathbf{3}_4$ and first and second dissociation for $\mathbf{3}_6$) are very low, (ii) the phenolate anion thus formed makes the cavity π-basic enough to include **2** or acetylcholine owing to the cation π-interaction,[3a,16] and (iii) the phenolate anion can act as a quencher for included **2**. We believe that the $\mathbf{3}_n$−**2** combined system has enabled us for the

(20) We evaluated whether other onium guests can regenerate the fluorescence intensity of **2**. At [onium guest]/[$\mathbf{3}_6$] = 6.0 we obtained the following I (in the presence of onium guest)/I_o (in the absence of onium guest) values and association constants (K_{ass}): I/I_o and K_{ass} are 1.25 and $10^{2.74}$ M^{-1} for $Et_2N^+Me_2OTs^-$, 1.60 and $10^{2.84}$ M^{-1} for $Et_3N^+HCl^-$, 1.69 and $10^{2.87}$ M^{-1} for $Me_3S^+ClO_4^-$, 1.70 and $10^{2.87}$ M^{-1} for $Et_3N^+MeClO_4^-$, and 1.70 and $10^{2.87}$ M^{-1} for $Et_4N^+ClO_4^-$. The error range for these K_{ass} values is $10^{\pm0.01}$. The results indicate that these onium guests can partially compete with **2** for the π-basic cavity but the I/I_o values are all smaller than that for acetylcholine (I/I_o = 2.70; K_{ass} = $10^{3.07}$ M^{-1}). Important from a practical viewpoint is the finding that as shown in Figure 3, this system is insensitive to biologically-ubiquitous ammonium groups in α-amino acids.

(21) In the $\mathbf{3}_6$−**4** complex the protons in **4** distinctly shifted as described in the text whereas those in $\mathbf{3}_6$ scarcely moved. The chemical shift changes ($\Delta\delta$) assuming 100% complexation were −0.83 ppm for N^+Me, −0.83 ppm for 3-H, and −0.54 ppm for 4-CH_3 (2-H was folded by Ar-H of $\mathbf{3}_6$). The N^+Me protons in acetylcholine also shifted to higher magnetic field ($\Delta\delta$ −1.04 ppm) in the presence of excess $\mathbf{3}_6$ (10-fold) where 100% complexation can be assumed.

first time to apply a convenient fluorescence method to the selective and nondestructive histochemical analysis of acetylcholine against amino acids in a biological system.

Experimental Section

Preparation of $\mathbf{3}_n$ ($n = 4$ and 6) has been described in well-known previous work and was carried out using the same process.[22] Compound **2** was synthesized as follows. 1-Pyrenecarboxaldehyde (0.46 g, 2.0 mmol) dissolved in THF (20 mL) was added to the MeOH solution containing 1,4-dimethylpyridinium iodide (0.47 g, 2.0 mmol) and piperidine (0.17 g, 2.0 mmol). After this mixture was stirred at room temperature for 6 h, the product precipitation was filtrated, dissolved in MeOH, and ion-exchanged by addition of Ag_2O and HCl. The reaction mixture was filtrated and the filtrate was evaporated to dryness: yield 74%, mp 245−249 °C dec; ^{1}H NMR (DMSO-d_6, 25 °C) δ 4.30 (CH_3, s, 3H), 7.85 and 9.12 (CH=CH, d, 1H each), 8.12−8.98 (pyrenyl-H, m, 13H). Anal. Calcd for $C_{24}H_{18}NCl$: C, 81.00; H, 5.10; N, 3.94. Found: C, 80.90; H, 5.07; N, 3.96.

The apparatus used for the measurement of ^{1}H NMR spectra was a JEOL GX-400 (400 MHz) spectrometer. The base-catalyzed methanolysis of acetylcholine was confirmed by a conventional HPLC method (column Zorbax ODS, mobile phase MeOH). Two peaks assignable to acetylcholine and methyl acetate appeared with a 82/18 ratio 2 h after the sample preparation.

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