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A New Fluorometric Method for the Detection of the Neurotransmitter Acetylcholine in Water Using a Dansylcholine Complex with *p*-Sulfonated Calix[8]arene

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Abstract

A new method for the fluorometric detection of the neurotransmitter acetylcholine (ACh) in water is presented. Use of the fluorescence of dansylcholine (DANCh) bound to *p*-sulfonated calix[8]arene affords a new fluorometric method for the detection of ACh ($>10^{-4}$ M) in aqueous solution (pH = 6.9). The fluorescence intensity of DANCh in aqueous solution was enhanced 1.8 fold after the complexation with *p*-sulfonated calix[8]arene. The addition of ACh to the aqueous solution of the DANCh-calix[8]arene complex significantly decreased the fluorescence intensity, which results from the replacement of DANCh in the complex with ACh. The effects of other synaptic neurotransmitters on the fluorescence of the DANCh complex were examined for dopamine, histamine, ATP, GABA, glycine, L-glutamic acid, and L-aspartic acid. Among the neurotransmitters studied, ACh was most effective in changing the fluorescence of the DANCh complex. Possible application of the DANCh complex dye for the detection of ACh in biological systems is discussed.

Introduction

Owing to the high sensitivity of the fluorometric method, a number of fluorescent sensor compounds have been developed for the measurement of pH, O_2 and concentrations of metal ions, such as Na^+, K^+, Ca^{2+} and Mg^{2+}, in biological systems [1]. For instance, the development of Ca^{2+} fluorescent probes [2] has contributed a great deal to the study of the signal transduction in the field of cell biology [3]. Now a variety of fluorescent probes for use in signal transduction studies are commercially available. However, little attention has been paid to the design of fluorescent probes for neurotransmitters, such as acetylcholine (ACh). ACh is one of the most abundant neurotransmitters in the nerve system [3]. There are many methods for its analysis: GC-MS [4] and HPLC [5] with chemiluminescence assay including the traditional approaches of bioassay [6], colorimetric assay [7], and radioimmunoassay [8]. However, these methods involve some complicated and time-consuming procedures, and require expensive equipment.

For the fluorometric detection of ACh, it is necessary to develop fluorescence labeling reagents for ACh or fluorescent probes that selectively respond to ACh against other neurotransmitters. ACh lacks a functional group to produce a fluorescent product. In addition, there are no reliable methods for the selective transformation of ACh into a fluorescent derivative in the presence of amino acids and monoamine neurotransmitters [9]. For these reasons, fluorescence labeling of ACh in biological systems is very difficult. Only Fellman has reported the fluorometric detection of ACh after conversion into acetyl hydrazine, which was reacted with salicyl aldehyde [10]. Recently, new fluorometric methods for the detection of ACh have been reported that are based on competition of ACh and a fluorescent analogue that is bound to a synthetic host molecule. Inouye et al. reported that the fluorescence of an ACh analogue, a pyrene-modified *N*-alkylpyridium cation, is useful for the selective detection of ACh in 0.01 M KOH/MeOH [11]. More recently, Shinkai and co-wokers have developed the detection method for ACh in water/MeOH using the fluorescence of a pyrene dye complex with *p*-sulfonated calix[6]arene [12]. However, these methods cannot be applied to the detection of ACh in physiological media: a mixed medium of water and methanol was used to suppress the aggregation of the pyrene dye observed in aqueous solution.

The aim of this work is to develop a simple fluorometric method for the detection of ACh in water. Herein, we report that the fluorescence of dansylcholine (DANCh) bound to *p*-sulfonated calix[8]arene (**3**) is useful for the determination of ACh concentration ($>10^{-4}$ M) in aqueous systems. The recognition mechanism of ACh by *p*-sulfonated calixarenes is studied on the basis of fluorescence and ^{1}H NMR spectra of DANCh complexes. Possible application of the DANCh-**3** complex for the detection of ACh in biological systems is discussed.

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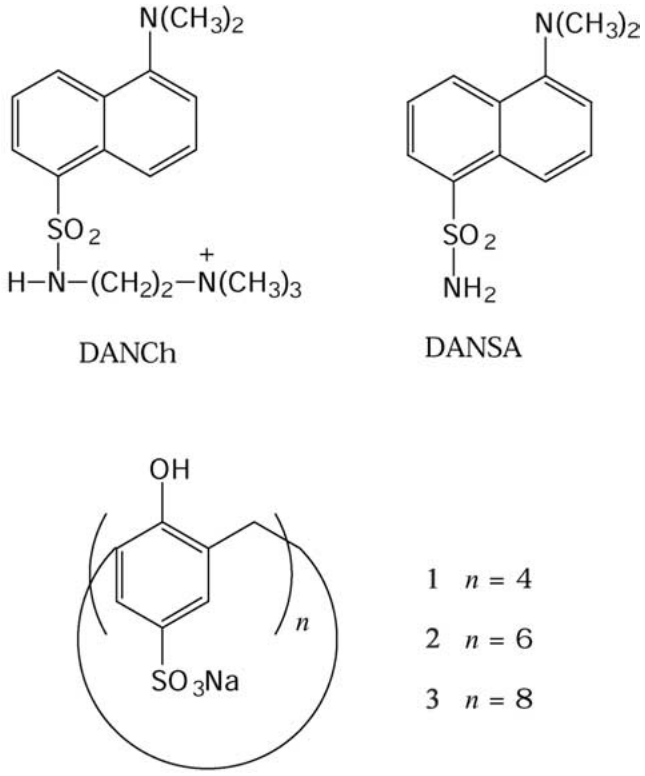


Scheme 1. Structures of dansylcholine (DANCh), dansylsulfonamide (DANSA) and *p*-sulfonated calix[n]arenes **1**, **2**, and **3**.

Experimental

Material and equipment

A fluorescent ACh analogue, dansylcholine (DANCh) [13], and dansylsulfonamide (DANSA) [14] were synthesized by the literature methods. Calix[4]arene *p*-sulfonic acid was purchased from Acros Organics (Belg.) and its sodium salt (**1**) was prepared from an aqueous solution (pH = 7) of the acid and NaOH. Sodium salts of *p*-sulfonated calix[6]- and calix[8]arene (**2** and **3**) were purchased from Dojin (Japan) and were used after recrystallization from water/methanol. Structures of the dansyl derivatives and *p*-sufonated calix[n]arenes are shown in Scheme 1. Neurotransmitter compounds (analytical reagent grade) including ACh were purchased from Wako Chemicals and were used without purification.

Fluorescence spectra measurements were performed with a Shimadzu RF-5300PC spectrofluorophotometer and quarts cuvettz (1 cm × 1 cm × 4.5 cm). Fluorescence spectra of dansyl derivatives were measured by excitation at 340 nm. All sample solutions for the measurements of fluorescence spectra were adjusted to pH = 6.9 using 25 mM phosphate (NaH_2PO_4—Na_2HPO_4) buffer. ^{1}H NMR spectra were measured using a Bruker XL-400 spectrometer operating at 400 MHz. Chemical shifts were referred to 3-(trimethylsilyl)-1-propanesufonic acid sodium salt (DSS, $\delta = 0$) as an internal standard. Deuterium oxide (99.9%) was purchased from Merck Co. All measurements were carried out at 25 °C.

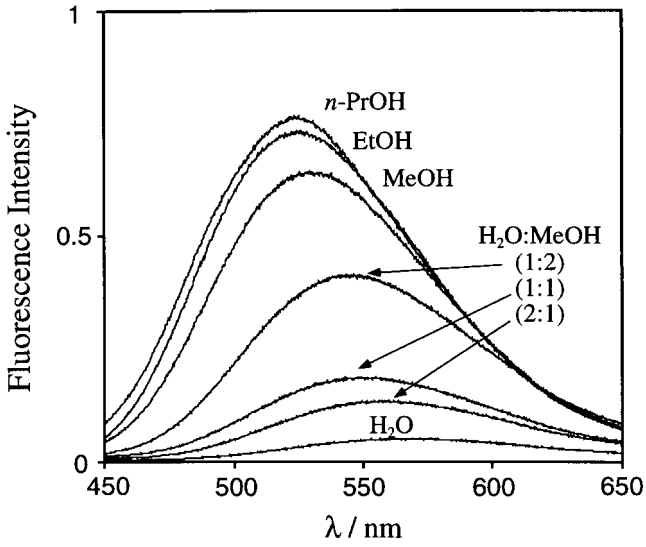


Figure 1. Effects of solvents on the fluorescence emission spectra of DANCh (3.3 μM).

Results and discussion

Fluorescence emission spectra of DANCh and DANSA

DANCh has been used as a fluorescent analogue of ACh, because it resembles ACh in the ethyltrimethylammonium end carrying a positive charge and binding to anionic sites in the acetylcholine receptor [13]. It has been shown that the fluorescence intensity of DANCh is enhanced by its binding to acetylcholine receptors [15]. The fluorescence enhancement of the DANCh is explained in terms of an increase in the hydrophobicity of its environment [15]. Figure 1 shows the solvent effect on the fluorescence spectra of DANCh with excitation at 340 nm. With decreasing polarity of the solvent, the fluorescence maximum shifts to shorter wavelengths, accompanied by a significant increase in fluorescence intensity. The emission maximum wavelengths of DANCh are 572 nm in H_2O, 560 nm in H_2O/MeOH (2 : 1), 549 nm in H_2O/MeOH (1 : 1), and 530 nm in MeOH. Thus the maximum is strongly influenced by the polarity of its surroundings. DANSA is also known to be a fluorescent probe that is sensitive to the polarity of solvents [14, 16]. The fluorescence spectrum of DANSA in water was almost the same as that of DANCh, except for difference in the fluorescence intensity. The fluorescence intensity of the DANSA in water was ca. 80% smaller than that of DANCh with the same concentration. The solvent effects on the fluorescence spectrum of DANSA showed the similar behavior with that of DANCh.

Fluorescence spectra of DANCh in the presence of p-sulfonated calix[n]arenas

Figure 2 shows the fluorescence spectra of DANCh (3.3 μM) in the presence of *p*-sulfonated calix[4]arene **1**, *p*-sulfonated calix[6]arene **2**, and *p*-sulfonated calix[8]arene **3** in aqueous solutions (pH = 6.9). All of the *p*-sulfonated calix[n]arenes were added directly as microlitre aliquots of 1 mM aqueous solutions to the DANCh solutions (3 ml). The changes in

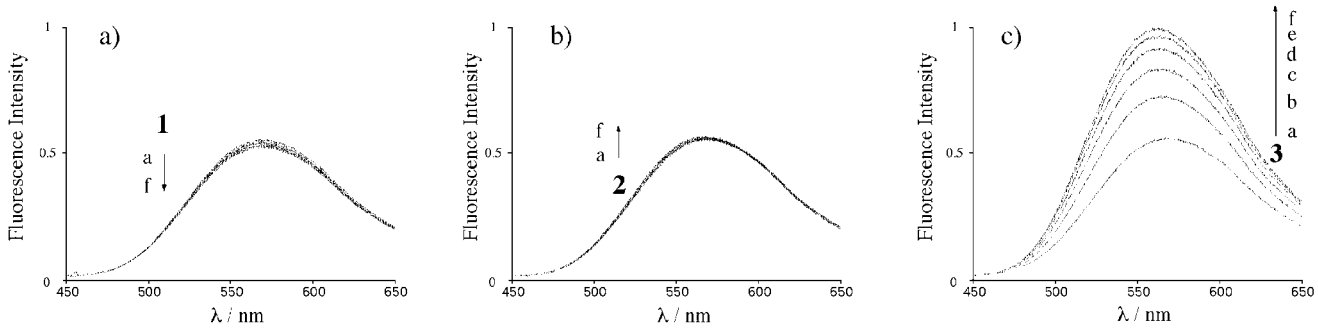


Figure 2. Fluorescence spectra of DANCh (3.3 μM) in the presence of *p*-sulfonated calix[n]arene sodium salts **1**, **2** and **3**: $a = 0$ μM, $b = 1.7$ μM, $c = 3.3$ μM, $d = 9.8$ μM, $e = 13.1$ μM, and $f = 19.5$ μM.

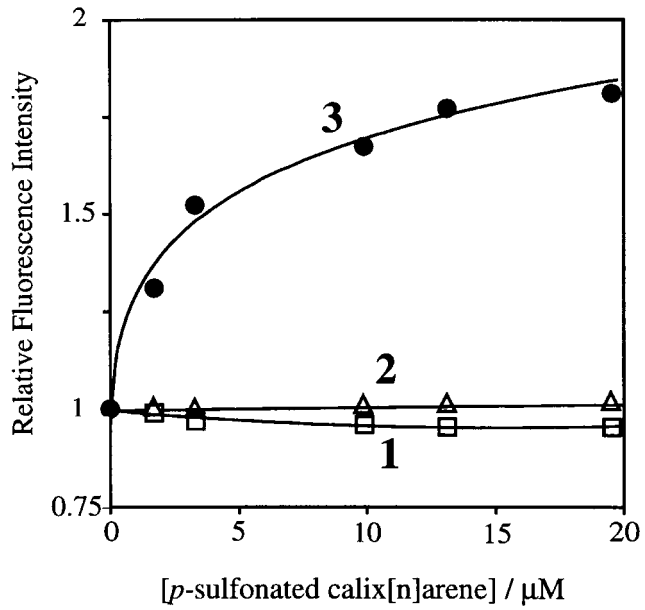


Figure 3. Changes in fluorescence intensity of DANCh (3.3 μM) at 560 nm upon the addition of *p*-sulfonated calix[n]arene sodium salts.

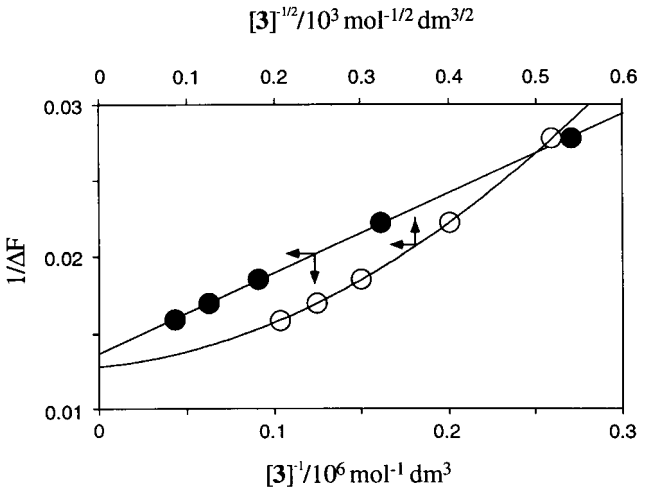


Figure 4. Plots of $1/\Delta F$ *versus* $[\mathbf{3}]^{-1}$ and $[\mathbf{3}]^{-1/2}$ at [DANCh] = 0.2 μM.

relative fluorescence intensity of the DANCh are shown in Figure 3. The addition of **1** and **2** to the DANCh solutions caused only a small spectral change. The fluorescence intensity of the DANCh decreased by a factor ca. 8% in the presence of a large excess amount of **1** (ca. 250 equiv.). In the case of the same large excess of **2**, ca. 3% of fluorescence enhancement was observed. The emission maximum of the DANCh was not shifted upon addition of **1** and **2**.

In contrast, the addition of **3** to the DANCh solution caused a significant enhancement of the fluorescence intensity: the intensity increased by a factor of 1.8 fold in the presence of 10 equiv. amounts of **3**. At the same time, a blue shift (from 572 nm to 558 nm) of the emission maximum of DANCh was observed. This result suggests that the dansyl moiety of DANCh is located in a more hydrophobic environment which is consistent with inclusion of DANCh within the aromatic cavity of **3**. Judging from the emission maximum (λ_{max}, 558 nm) of the DANCh-**3** complex, the surrounding environment of the dansyl moiety has a similar polarity as a 2 : 1 mixture of H_2O/MeOH.

The stoichiometry and dissociation constant of the DANCh complex with p-sulfonated calix[8]arene

The stoichiometry of the DANCh-**3** complex and its dissociation constant K_{DANCh} can be determined by analysis of the fluorescence intensity change (ΔF) [17]:

$$1/\Delta F = 1/a + K_{\mathrm{DANCh}}/a[\mathbf{3}]^{1/n}, \qquad (1)$$

where ΔF is an intensity difference of the DANCh fluorescence in the absence and presence of **3**, n is a number of DANCh molecules bound to **3**, and a is a constant. If a single DANCh molecule is contained in the DANCh-**3** complex, a plot of $1/\Delta F$ *versus* $1/[\mathbf{3}]$ will be linear when **3** is in excess. Binding of two DANCh molecules to **3** will lead to a linear plot of $1/\Delta F$ *versus* $1/[\mathbf{3}]^{1/2}$ under the same condition. These plots are shown in Figure 4. The plot of $1/\Delta F$ *versus* $1/[\mathbf{3}]$ shows a good linear relationship ($r = 0.99$). In contrast, the plot of $1/\Delta F$ *versus* $1/[\mathbf{3}]^{1/2}$ is non-linear. This result verifies the formation of a 1 : 1 complex:

$$\mathrm{DANCh} + \mathbf{3} \rightarrow \mathrm{DANCh\text{-}}\mathbf{3}. \qquad (2)$$

The dissociation constant K_{DANCh} of DANCh-**3** complex in aqueous solution at pH = 6.9 is calculated to be 3.0 μM. For the DANCh-**1** and DANCh-**2** complexes, dissociation constants could not be determined because of the very small fluorescence changes.

Table 1. Chemical shifts of the protons of $—N^+Me_3$ and $—NMe_2$ of DANCh

Sample	Chemical shifts (ppm)	
	$—N^+(CH_3)_3$	$—N(CH_3)_2$
DANCh	3.09	2.90
DANCh + **1**	1.10	2.90
DANCh + **2**	1.74	2.93
DANCh + **3**	1.81	2.10

[DANCh] = 5 mM, [calix[n]arene] = 12.5 mM, pD = 6.9 adjusted by 25 mM phosphate buffer.

NMR evidence for the complexation of DANCh with p-sulfonated calix[n]arenas

To provide insight into the binding of DANCh by *p*-sulfonated calix[n]arenes, ^{1}H NMR spectra of DANCh in the presence of **1**, **2**, and **3** were examined. Table 1 shows the chemical shifts of the quaternary ammonium moiety ($—N^+Me_3$) and the dimethylamine unit ($—NMe_2$) of DANCh in the absence and presence of excess amounts (2.5 equiv.) of *p*-sulfonated calix[n]arenes. The proton signal of the quaternary ammonium moiety is strongly shifted upfield by addition of **1**, **2**, and **3**. The order of magnitude of the change in chemical shifts was **1**> **2**> **3**>. These shifts reveal that all three *p*-sulfonated calix[n]arenes interact with the quaternary ammonium moiety of DANCh in aqueous solution. Similar upfield shifts of the quaternary ammonium moiety have been reported in systems of ACh + resorcin[4]arene and ACh + *p*-sulfonated calix[n]arenes (n = 4 and 6) [18]. It should be noted that the addition of **3** also shifted the proton signal of the dimethylamine moiety to higher magnetic field from 2.90 ppm to 2.10 ppm, but the addition of **1** and **2** did not. This finding suggests that the dansyl moiety is included within the calixarene cavity in the case of **3**.

Evidence for the inclusion of dansyl moiety by **3** is also provided by the ^{1}H NMR spectra of the aromatic protons of DANCh (Figure 5). The aromatic proton signals in the dansyl moiety are changed significantly by the addition of **3**. Again, this shows that the dansyl moiety is included in the π-basic cavity [12, 18, 19] created by the benzene units of **3**. Greater sensitivity of the chemical shifts of 6′- and 7′-H on complexation indicates that this part of dansyl moiety is more deeply inserted into the cavity of **3**. For **1** and **2**, the changes in ^{1}H NMR spectra are much smaller than that observed in **3**, suggesting that the dansyl moiety is not deeply included by the calixarene cavity. In fact, the proton signal for the dimethylamine moiety shifts very little in the presence of **1** and **2** (Table 1). On the basis of the ^{1}H NMR and the fluorescence spectral data (Figure 2), schematic representations for the DANCh complexes with **1**, **2**, and **3** are shown in Scheme 2. The difference in the binding modes may be attributed to the size of the calixarene cavity. The smaller cavity calixarenes **1** and **2** probably do not have a sufficient cavity space to accommodate the dansyl group and the quaternary ammonium group at the same time.

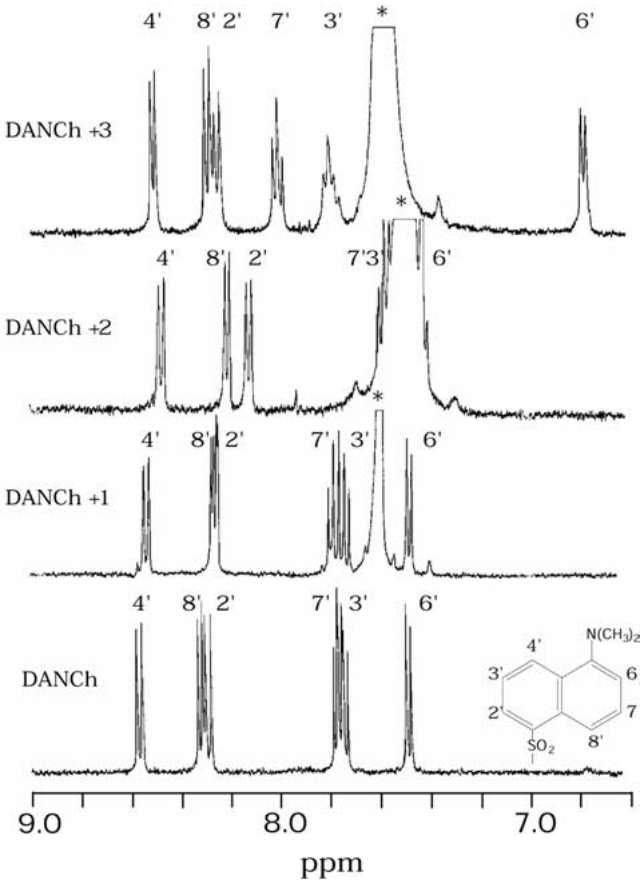
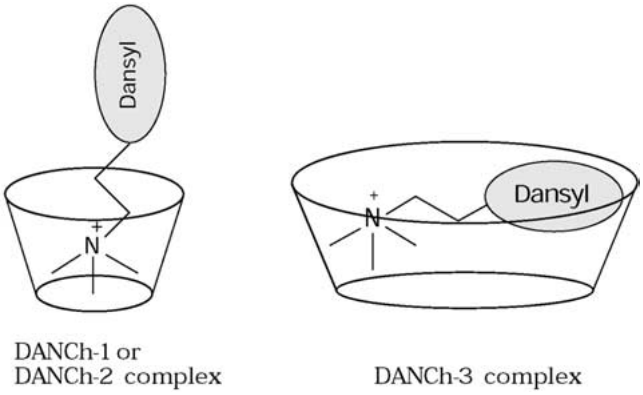


Figure 5. ^{1}H NMR spectra of the aromatic region of DANCh in D_2O in the absence and presence of *p*-sulfonated calix[n]arene sodium salt. Signals marked with asterisk arise from the aromatic protons of the calix[n]arenes. The conditions were the same as those given in Table 1.



Scheme 2. Schematic representation for the DANCh complexes with **1**, **2**, and **3**.

Driving force for inclusion of DANCh by p-sulfonated calix[8]arene

Since the guest molecule, DANCh, has both an ammonium cation group and a hydrophobic dansyl moiety, it is expected that both electrostatic force and a hydrophobic effect will play a role in the inclusion of DANCh by **3**. To probe the driving force for inclusion of DANCh by **3**, we examined whether a neutral dansyl derivative DANSA can be included by **3**. If DANSA is included by **3**, the main driving force should be a hydrophobic effect arising from the

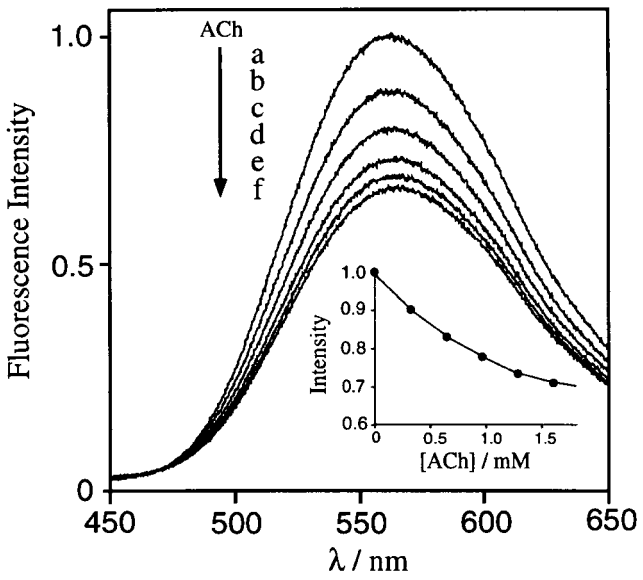


Figure 6. Fluorescence spectra of the DANCh-**3** complex ([DANCh] = 3.3 μM and [**3**] = 16 μM) in the presence of ACh: a = 0 mM, b = 0.32 mM, c = 0.65 mM, d = 0.97 mM, e = 1.3 mM, and f = 1.6 mM. The inserted figure shows the dependence of the fluorescence intensity of DANCh on the ACh concentration.

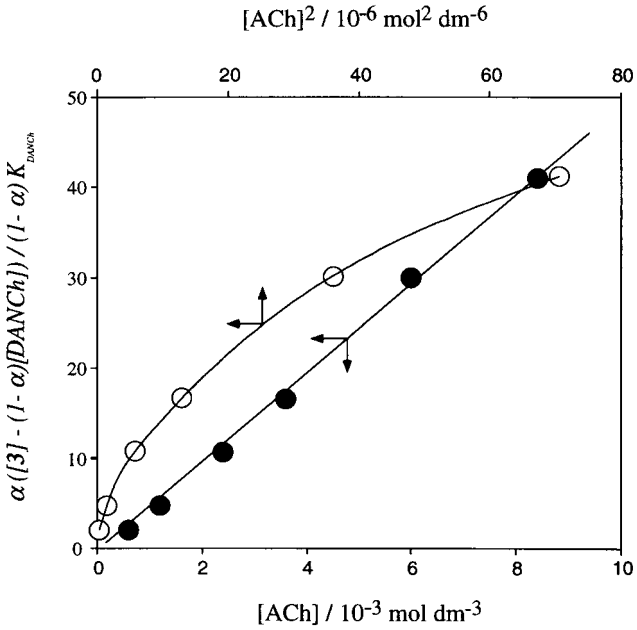


Figure 7. Plots of $\alpha([\mathbf{3}]-(1-\alpha)[\text{DANCh}])/(1-\alpha)K_{\text{DANCh}}$ *versus* [ACh] and $[\text{ACh}]^2$ at [DANCh] = 1 μM and [**3**] = 24.4 μM.

dansyl moiety, not an electrostatic interaction between the quaternary ammonium cation and the electron rich aromatic ring of **3**. Addition of excess amounts (50 equiv.) of **3** to an aqueous solution of DANSA (3.3 μM) did not change the fluorescence spectrum, reveals that **3** cannot include a neutral DANSA molecule. Similar results were obtained with other environment-sensitive probes, such as PRODAN [20] (6-propionyl-2-dimethyl-aminonaphthalene) and ANS [21] (1-anilino-8-naphthalene sulfonic acid). These results clearly demonstrate that the main driving force for inclusion of DANCh by **3** is electrostatic interaction (cation-π interaction) [12, 18, 19, 22] between the quaternary ammonium cation and the π-basic cavity created by benzene units. Indeed, other cationic fluorescent molecules, such as Rhodamine 6G and Rhodamine 123, can also complex with **3** [23].

*Fluorometric detection of ACh based on competitive binding of ACh to the DANCh-**3** complex*

Figure 6 shows the fluorescence change in the DANCh-**3** complex by addition of ACh. The emission intensity of the dansyl fluorescence decreased with increasing ACh concentration. Thus, DANCh bound to **3** is replaced with ACh:

$$[\text{DANCh-}\mathbf{3}] + \text{n}\cdot\text{ACh} \rightarrow [\text{n}\cdot\text{ACh-}\mathbf{3}] + \text{DANCh}. \quad (3)$$

In the presence of excess amounts (600 equiv.) of ACh, the fluorescence intensity decreased by a factor of 30%. The inserted figure shows dependence of the relative fluorescence intensity at 560 nm on the ACh concentration. This data suggests that the fluorescence response of the DANCh-**3** complex is useful for detection of ACh at $>10^{-4}$ M levels in aqueous solution.

For competitive binding of ACh to the DANCh-**3** complex with a dissociation constant K_{ACh}, the relationship between two dissociation constants (K_{ACh} and K_{DANCh}) is expressed by the following equation under the condition of ACh in excess [24]:

$$\alpha([\mathbf{3}]-(1-\alpha)[\text{DANCh}])/(1-\alpha)K_{\text{DANCh}} = 1+[\text{ACh}]^n/K_{\text{ACh}} \quad (4)$$

$$\alpha = (F_0 - F)/(F_0 - F_\infty), \quad (5)$$

where F_0, F, and F_∞ are the maximum intensities for the dansyl emission measured before, during, and after the titration with ACh to a saturation concentration. Figure 7 shows the plot of $[\text{ACh}]^n$ *versus* α $([\mathbf{3}] - (1-\alpha)[\text{DANCh}])/(1-\alpha)K_{\text{DANCh}}$. As can be seen from Figure 7, the plot shows a linear relationship (r = 0.98) when n is set to be 1, indicating that one ACh molecule complexes with **3**:

$$[\text{DANCh-}\mathbf{3}] + \text{ACh} \rightarrow [\text{ACh-}\mathbf{3}] + \text{DANCh}. \quad (6)$$

From the slope of the plot, the dissociation constant (K_{ACh}) for the ACh complex with **3** was calculated to be 182 μM. This result shows that the affinity of ACh for **3** is lower than that of DANCh by a factor of ca. 60. The higher binding affinity of DANCh for **3** may be attributed to a hydrophobic effect arising from the aromatic moiety of DANCh.

*Possible application of DANCh-**3** complex for the detection of ACh in biological systems*

Fluorescence response of DANCh-**3** complex was examined for several synaptic neurotransmitters, including ACh and its hydrolyzed product, choline. Figure 8 shows the percent

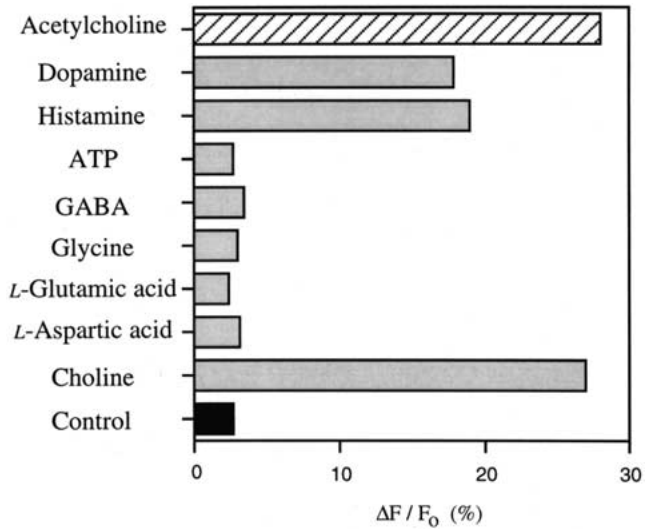


Figure 8. Decrease ($\Delta F/F_0$) in fluorescence intensity of DANCh-**3** complex ([DANCh] = 3.3 μM and [**3**] = 16 μM) at 560 nm in the presence of neurotransmitters and choline (1.6 mM). Microlitre aliquots (50 μl) of neurotransmitter solutions (100 mM) were added to the DANCh-**3** solution (3 ml). A control experiment was performed in which only 50 μl of 25 mM phosphate buffer was added to the DANCh-**3** solution.

of decrease in the fluorescence intensity of the DANCh-**3** complex (ca. 3 μM) in the presence of excess amounts (1.6 mM) of the neurotransmitters and choline. Among the neurotransmitters, ACh is most sensitive to change in the fluorescence intensity. In the presence of ACh, the fluorescence intensity decreased by 28%. Addition of dopamine and histamine also decreased the intensity by 17% and 18%, respectively. This finding indicates that *p*-sulfonated calix[8]arene **3** can bind the primary ammonium moieties of dopamine and histamine, as well as the quaternary ammonium moiety of ACh. Interestingly, the fluorescence intensity of the DANCh-**3** complex was scarcely affected by addition of ATP, GABA, and amino acid neurotransmitters (such as glycine, L-glutamic acid, and L-aspartic acid). This observation is consistent with the previously described lack of binding of **3** toward neutral and anionic host molecules, such as PRODAN and ANS. Furthermore, we examined fluorescence spectrum of DANCh-**3** complex in the presence of KCl, NH_3Cl and other amino acids (such as L-alanine and L-phenylalanine). Addition of large amounts (600 equiv.) of these chemicals to an aqueous solution of DANCh-**3** complex (ca. 3 μM) did not change the fluorescence spectrum. These results show that the DANCh-**3** complex can be used for detection of ACh and monoamine neurotransmitters, such as dopamine and histamine, in the presence of ATP and amino acids in physiological salt solution.

From the view of application to biological tissues, however, this ACh detection method using the DANCh-**3** complex has several problems. Firstly, the sensitivity ($>10^{-4}$ M) in this ACh detection system is insufficient for the accurate determination of ACh at levels found in biological tissues. For example, the concentration of ACh in the synaptic cleft is ca. 5×10^{-4} M [3]. Secondly, this detection system cannot distinguish between ACh and choline (Figure 8). In the nerve systems, ACh is rapidly hydrolyzed to choline and acetic acid by a specific enzyme (acetylcholine esterase): deactivation of acetylcholine esterase is usually performed by microwave irradiation for the determination of ACh in biological tissues [25]. Thirdly, fluorescence measurements using the DANCh-**3** complex have to be carried out by excitation at around 340 nm. At these wavelengths, a number of intrinsic fluorophores, such as NAD(P)H, in biological tissues would be excited at the same time [26]. Thus, for the application to biological systems, the sensitivity, selectivity, and excitation wavelengths of this detection system should be improved by modification of the fluorescent ACh analogue and the host molecule.

Conclusion

We have presented a novel strategy for the fluorometric detection of the neurotransmitter ACh in water. The fluorescent ACh analogue DANCh binds *p*-sulfonated calix[8]arene (**3**) to form a 1 : 1 complex of DANCh-**3**. Competitive binding of ACh to the DANCh-**3** complex significantly changes the emission intensity of the dansyl fluorescence. The fluorescence of the DANCh is most sensitive to ACh among the synaptic neurotransmitters, dopamine, histamine, ATP, GABA, glycine, L-glutamic acid, and L-aspartic acid. In addition, the fluorescence of the DANCh-**3** complex is not changed in the presence of K^+, NH_3^+ ions, ATP, and amino acids. The present study affords a new fluorometric method for the determination of ACh ($>10^{-4}$ M) in the presence of ATP and amino acids in physiological salt solution. The design of more efficient and selective ACh detection system using *p*-sulfonated calix[8]arene is now in progress for the application to biological systems.

Acknowledgments

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