Detection of Charge Movements in Ion Pumps by a Family of Styryl Dyes

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Abstract. A family of fluorescent styryl dyes was synthesized to apply them as probes that monitor the ion-translocating activity of the Na,K-ATPase and the SR Ca-ATPase, similar to the widely used dye RH421. All dyes had the same chromophore but they differed in the length of the spacer between chromophore and polar head, an isothiocyanate group, and in the lengths of the two identical acyl chains, which form the tail of the dye molecules. A number of substrate-dependent partial reactions of both P-type ATPases affected the fluorescence intensity, and the magnitude of the fluorescence changes was used to characterize the usefulness of the dyes for further application. The experimental results indicate that electrochromy is the major mechanism of these dyes. While in the case of the Na,K-ATPase a single dye, 5QITC, showed larger fluorescence changes than all others, in the case of the SR Ca-ATPase all dyes tested were almost equal in their fluorescence responses. This prominent difference is interpreted as a hint that the position of the ion binding sites in both ion pumps may differ significantly despite their otherwise closely related structural features. Quench experiments with spin-labeled lipids in various positions of their fatty acids were used to gain information on the depth of the chromophore of the different dyes within the membrane dielectric, however, the spatial resolution was so poor that only qualitative information on the position of the chromophore in the lipid phase could be obtained.

Key words: P-type ATPases — Ion binding sites — Electrochromic effects — Styryl dyes — Fluorescence — Partial reactions

Introduction

Detailed investigations of the ion-transport process in P-type ATPases have revealed pump cycles in which the ions are moved across the membranes by several reaction steps. Most of these pumps transfer different ions in opposite directions, such as Na^+ vs. K^+, H^+, vs. K^+ or Ca^{2+} vs. H^+, and in all these cases the process is a so-called Ping-Pong mechanism (Läuger, 1991). Each "half cycle" consists of an ordered sequence of experimentally identified steps: ion binding, ion occlusion (together with enzyme phosphorylation or dephosphorylation), transition between both principal conformations, $E_1 \rightarrow E_2$ or vice versa, ion deocclusion and release to the aqueous phase on the other side of the membrane. In principle, the ions could be moved through the interior of the protein dielectric to a certain extent at each of these steps; however, recent investigations revealed that a charge movement occurred mainly during the ion binding and release steps (Apell, Schneeberger & Sokolov, 1998; Butscher, Roudna & Apell, 1999; Apell & Karlish, 2001). Those reaction steps are termed "elctrogenic" (Läuger & Apell, 1989; Apell, 1989).

To detect and to analyze such reaction steps, experimental techniques are necessary, which allow a resolution of the charge movement in time and amplitude. In the case of the Na,K-ATPase, during the last decade two different approaches were applied, which both produced kinetical information on electrogenic partial reactions. The first approach made use of direct electric measurements, either by electrophysiological techniques applied to native cells (Rakowski et al., 1990, Gadsby, Rakowski & De Weer, 1993; Rakowski, 1993; Hilgemann, 1994; Holmgren et al., 2000) or by use of capacitive coupling of open membrane fragments purified from kidney tissue, which were adsorbed tightly to planar lipid bilayers (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987; Wuddel & Apell, 1995; Sokolov et al., 1998;

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Pintschovius, Fendler & Bamberg, 1999; Domasze-
wicz & Apell, 1999). As an alternative method an
indirect approach was introduced, which utilizes
electrochromic styryl dyes that allow the detection of
charge movements in the Na,K-ATPase (Klodos &
Forbush, III, 1988; Bühler et al., 1991; Stürmer et al.,
1991; Pratap & Robinson, 1993; Heyse et al., 1994;
Fedosova, Cornelius & Klodos, 1995; Klodos, Fe-
dosova & Cornelius, 1997; Clarke et al., 1998;
Schneeberger & Apell, 1999). In the case of the Ca-
ATPase of the sarcoplasmatic reticulum (SR) the
situation is different: direct electric measurements are
not possible since the membrane of the SR is
extremely leaky for monovalent cations, a property
that short-circuits both sides of the membrane.
Therefore, investigations to identify and analyze the
electrogenic reaction steps with native membranes
containing the SR Ca-ATPase so far could be per-
formed successfully only with the use of styryl dyes
(Butscher et al., 1999; Peinelt & Apell, 2002). Ori-
ginally, styryl dyes were used to monitor fast changes
of transmembrane electric potentials (Loew et al.,
1979; Loew & Simpson, 1981; Loew, 1982; Grinvald
et al., 1983, 1987; Ehrenberg, Meiri & Loew, 2000).
The proposed mechanism of the styryl dyes is based
mainly on the effect of electrochromy, by which
changes of the (local) electric field inside the mem-
brane dielectric shift the absorption spectra and thus
affect the fluorescence yield when the chromophore is
excited at a wavelength preferentially at the red edge
of its absorption spectrum (Loew et al., 1979; Fluhler
et al., 1985; Bühler et al., 1991). Besides the electro-
chromic shift of the absorption spectrum of styryl
dyes, their voltage response may depend on addi-
tional components, which also affect the excitation
process of the dye molecules, as was discussed by
several authors (Fluhler et al., 1985; Müller, Wind-
isch & Tritthart, 1986; Ephardt & Fromherz, 1989;
Fedosova et al., 1995).

The styryl dye molecules are strongly hydro-
phobic with partition coefficients $\gamma = c_{lipid}/c_{water} > 10^5$
(see below), they have an amphiphilic nature and are
statistically distributed within the lipid leaflet of the
membrane. Due to the polar head group and the
hydrophobic chromophore and tail the dye molecules
are inserted in an oriented fashion with the polar or
charged head sticking in between the lipid head
groups and the alkyl chains in between the hydro-
phobic core of the lipids.

To study charge-translocating reaction steps in
the reaction sequence of an ion pump with such
electrochromic dyes, it is crucial to have a high
density of proteins in the membrane, in which the
number or position of ionic charges inside the
transmembrane proteins is changed by the transport
process. This requirement can be explained by basic
physical principles. On the one hand, the electro-
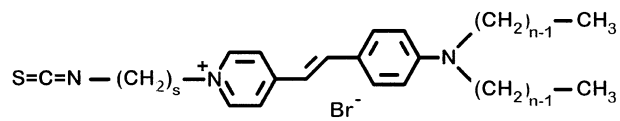chromic effect of a fluorescent dye is proportional to

Fig. 1. Principal structure of the fluorescent styryl dyes of the
nXITC family. The spacer length between the polar isothiocyanate
group and the chromophore, $(CH_2)_s$, was chosen between 3 and 6,
and is represented by the first letter of its name: PITC ($s = 3$),
BITC ($s = 4$), QITC ($s = 5$), HTTC ($s = 6$). The hydrophobic tail
consists of two alkyl chains with n carbons, $1 \leq n \leq 7$. The length of
the chain is indicated by the number in front of the letters.

the electric field it senses. On the other hand, if we,
for the sake of simplicity, treat the protein-containing
membranes as a plate capacitor with a homogeneous
and low dielectric constant, ε (in the order of 2–4), the
electric field is proportional to the charge density, σ.
The more protein molecules per membrane area bind
or release ions, the greater is the change of σ, the
larger is the change of the electric field, and, in con-
sequence, the stronger is the response of the fluor-
escent dyes. Under the assumption of a homogeneous
membrane with a thickness of 5 nm, a dielectric
constant $\varepsilon = 3$, and a pump density of 10,000 pumps
per μm^2, the charge density σ would be 1.6×10^{-3} C/
m^2. The corresponding amplitude of the electric field
is then calculated to be $E = 6 \times 10^7$ V/m. This is
about three times the amplitude of the electric field
generated by a transmembrane potential of 100 mV.

Various fluorescent styryl dyes produced differ-
ent responses in different membrane/ion pump sys-
tems. The chromophore itself as well as the
hydrophobic tail and the spacer between the chro-
mophore and polar "head" of the dye are known to
affect the sensitivity of the fluorescence response on
charge-translocating reaction steps of the ion pumps
(Fedosova et al., 1995, Butscher et al., 1999). This
observation led to the conception of this paper to
study systematically the effect of charge movements
produced by the action of the Na,K-ATPase and the
Ca-ATPase on the fluorescence responses of a family
of styryl dyes, named nXITC, with various lengths of
the hydrophobic tail and of the spacer between polar
head and chromophore (Fig. 1).

Materials and Methods

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase,
NADH, ATP (disodium salt, special quality) were from Boehringer
(Mannheim, Germany). Tricine and TEMPO (2,2,6,6-Tetra-
methylpiperidine-N-oxyl) were purchased from Sigma (Munich,
Germany). Sodium dodecylsulfate (SDS) was obtained from
Pierce Chemical (Rockford, IL). Dioleoylphosphatidyl choline
(DOPC), TEMPO-PC and 5-, 7-, 10-, 12-, and 16-doxyl-l-palmi-
toyl-2-stearoyl-sn-glycero-3-phosphocholine were purchased from
Avanti Polar Lipids, Alabaster, Ala. RH 421 (N-(4-sulfobutyl)-4-
(4-(4-(dipentylamino) phenyl-butadienyl-pyridinium, inner salt),

Table 1. UV/VIS data of the synthesized isothiocyanate dyes, nXITC, dissolved in EtOH

X	s	λ_{max} / nm (ε / 10^3 M^{-1} cm^{-1})						
		R = CH_3 $n = 1$	C_2H_5 $n = 2$	C_3H_7 $n = 3$	C_4H_9 $n = 4$	C_5H_{11} $n = 5$	C_6H_{13} $n = 6$	C_7H_{15} $n = 7$
P	3	490 (45.1)	504 (50.9)	504 (53.7)	505 (52.7)	506 (38.0)	499 (30.2)	506 (30.7)
B	4	488 (44.2)	500 (48.6)	502 (50.8)	503 (52.5)	503 (47.0)	496 (26.0)	503 (46.4)
Q	5	487 (39.8)	499 (50.2)	498 (54.3)	501 (54.9)	501 (41.7)	496 (26.9)	502 (33.7)
H	6	485 (49.0)	498 (49.7)	500 (54.4)	500 (56.5)	500 (49.0)	496 (28.6)	500 (30.7)

EGTA and Bis-Tris (Bis(2-hydroxyethyl)amino-tris (hydroxymethyl) methane) were obtained from Fluka (Buchs, Switzerland). NaCl (suprapure quality) and all other reagents (at least analytical grade) were from Merck (Darmstadt, Germany).

PREPARATION OF MEMBRANE FRAGMENTS CONTAINING Na, K-ATPase

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys in the form of open membrane fragments using procedure C of Jørgensen (Jørgensen, 1974). The protein concentration was assessed by the Lowry method, using bovine serum albumin as a standard. Specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971). The specific activity of the preparations used was in the range of 1,900 to 2,300 μM P_i per mg protein and hr at 37°C.

PREPARATION OF SARCOPLASMIC RETICULUM MEMBRANES CONTAINING Ca-ATPase

SR-Ca-ATPase was prepared by a slight modification of the method of Heilmann et al. (1977) from psoas muscle of rabbits. The whole procedure was performed at temperatures below 4°C. The determination of the protein content of the membrane preparation was performed according to Markwell et al. (1978). The most active fractions of the final density-gradient separation had a protein content of 2–3 mg/ml. The enzymatic activity was determined by the linked pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971) in a buffer containing 2 μM of free Ca^{2+}. Background enzymatic activity of the isolated preparation was obtained by either quantitative removal of the free Ca^{2+} by 5 mM EGTA or addition of 1 μM tharpsigargin. The Ca-ATPase-specific activity was about 1.8 units/mg at 20°C and pH 7.5 (which corresponds to 1.8 μmol ATP hydrolyzed per mg protein per min) and could be increased up to 2.6 units/mg in the presence of A23187 to short-circuit the membranes for Ca^{2+}.

SYNTHESIS OF THE FAMILY OF STYRYL DYES

The styryl dyes were prepared by the following general procedure (Hassner et al., 1984; Birmes, 1995): A mixture of 4-bromo-N,N-di-n-alkylaniline (22.5 mmol), 4-vinylpyridine (30 mmol), palladium diacetate (0.45 mmol), and tri-o-tolylphosphine (0.9 mmol) in dry triethylamine (18 ml) was heated at 110°C for 72 hr under argon. Water (200 ml) and chloroform (300 ml) were added to the cooled mixture. The chloroform phase was separated and the water layer was extracted with chloroform (3×50 ml). The combined chloroform solutions were washed with water, dried over $MgSO_4$ and evaporated to give the 4-(p-N,N-di-n-alkylamino)-styrylpyridine. Purification of the product was carried out by recrystallization from an appropriate solvent (e.g., n-pentane).

A solution of 4-(p-N,N-dialkylamino)-styrylpyridine (3 mmol) and ω-bromo-n-alkyl-isothiocyanate (9 mmol) in dry acetone (2 ml)

was refluxed. The quaternization progress was monitored by thin layer chromatography (TLC) using chloroform as eluent. After completed conversion the red solid was separated and recrystallized from acetone/n-hexane. The products were identified by ^{1}H-NMR spectroscopy and IR spectroscopy. The IR spectra of all dyes show the characteristic $n(N = C = S)$ band at 2200–2000 cm^{-1}. The purity was checked by TLC.

The ω-bromo-n-alkyl-isothiocyanates were synthesized using a procedure by P. Friis (1965): ω-Bromo-n-alkylamine hydrobromide (171 mmol) was treated with thiophosgen (170 mmol) in chloroform (80 ml) and triethylamine (600 mmol) and was then isolated by distillation under reduced pressure.

The spectroscopic data of the resulting dyes are shown in Table 1.

FLUORESCENCE EXPERIMENTS

Fluorescence measurements were performed with a commercial fluorescence spectrometer LS 50B (Perkin Elmer, Überlingen, Germany) with quartz cuvettes of 1 or 2 ml effective volume. A 515 nm cut-off filter in the emission pathway was used for all measurements. The cuvette holder was thermostated at 20°C and equipped with a magnetic stirrer. Experiments to study ion-pump specific responses with the styryl dyes were performed as so-called standard experiments, which were described previously in the case of Na, K-ATPase (Schneeberger & Apell, 1999) and of SR Ca-ATPase (Butscher et al., 1999). In a first set of experiments, the excitation wavelength dependence of the fluorescence signals was recorded in steady-state conditions of the Na, K-ATPase and of the Ca-ATPase, which could be maintained by defined substrate compositions of the ion pumps. In a second set of such experiments, the time course of the fluorescence intensity was investigated at the optimum wavelength combination for excitation and emission, and the fluorescence changes of well-defined partial reactions were detected, which could be triggered by successive addition of substrates. The concentration of the dyes applied was typically 200 nM. The excitation and the emission wavelengths for the applied dyes are listed for both ion pumps in Tables 3 and 4. The slit widths were 5 or 7.5 nm for the excitation and 10 or 15 nm for the emission monochromator.

DETERMINATION OF PARTITION COEFFICIENT OF THE STYRYL DYES

Lipid vesicles were prepared from synthetic dioleoylphosphatidyl choline (DOPC) by a dialysis method producing homogeneous unilamellar vesicles with an average diameter of about 80 nM as described previously (Apell et al., 1985). The membrane-water partition coefficient, γ, of the dye is defined by $\gamma = c_1/c_w$, where c_1 and c_w are the respective dye concentration in the lipid phase and water phase. The experiments were performed as described previously (Apell & Bersch, 1987).

Table 2. Partition coefficients of selected nXITC dyes determined with DOPC vesicle preparations

X	s	Partition coefficient $\gamma = c_{\text{lipid phase}} / c_{\text{water phase}}$			
		C_2H_5 $n = 2$	C_4H_9 $n = 4$	C_5H_{11} $n = 5$	C_7H_{15} $n = 7$
P	3	38,000	284,000	336,000	
B	4	45,000	544,000	654,000	1,614,000
Q	5	45,000	711,000	804,000	
H	6			638,000	1,716,000

PREPARATION OF LIPID VESICLES CONTAINING DOXYL LABELS AND STYRYL DYES

Vesicles for quenching experiments were prepared by sonification. They were composed of lipid (either 100% DOPC or 85% DOPC and 15% of one of the spin-labeled phospholipids) and 200 mM of one of the styryl dyes. Lipids and dyes were dissolved in organic solvent (ethanol, methanol and chloroform for dyes, DOPC, and spin-labeled lipids, respectively). Dye, DOPC, and spin-labeled lipid were added into a test tube, and the organic solvents were evaporated under a flow of argon. Afterwards, the samples were dried in vacuum for 45 minutes. Then 2 ml buffer (25 mM histidine, 100 mM NaCl, 5 mM MgCl$_2$, and 0.5 mM EDTA, pH 7.2) were added, and the samples were sonicated for 3 minutes in an ultra-sonication bath (Transsonic Digital, Elma, Singen, Germany).

During sonication the samples were kept under a constant flow of argon to prevent oxidation. The final lipid concentration of the samples was checked by the Phospholipids B test (Wako Chemicals, Neuss, Germany) to be 60 μM pure DOPC or DOPC with 15% spin-labeled phospholipid. With a dye concentration of 200 nM, the (molar) lipid/dye ratio was 300:1.

EPR MEASUREMENTS TO DETERMINE THE CONCENTRATION OF DOXYL LABELS

For the accuracy of the quench experiments it was crucial to maintain a constant concentration of the quenching spin labels and of the same ratio of dye/lipid/spin label in all vesicle preparations. To determine the concentration of spins in the labeled lipids, EPR analysis was performed. The intensities of the doubly integrated EPR spectra of the doxylated phospholipids were compared to those of a defined TEMPO standard. Duplicate samples of spin-labeled lipids dissolved in DMSO were prepared. The EPR spectrometer was calibrated with TEMPO samples at concentrations between 80 and 400 μM in DMSO. The samples were pipetted into 100 μl capillaries sealed with sealing wax (Critoseal, Oxford Labware, Chicago, IL) and placed in the sample cavity of a Varian E 109 spectrometer (Varian, Darmstadt, Germany). The microwave power was 2 mW in order to avoid signal saturation, the magnetic field was scanned between 3325 and 3425 Gauss, and the detector current was 250 μA. The spectra were analyzed with the computer program "EPR" by Frank Neese, University of Konstanz, and the values of the doubly integrated spectra were used to determine the spin concentration. The ratios of the spin/lipids in the purchased stock solutions of spin-labeled phospholipids were found to be in the range of 0.75 to 0.95.

The concentration of TEMPO-PC was checked by a phosphate assay (Bartlett, 1959) subsequent to total digestion of the lipid (Morrison, 1964).

FLUORESCENCE QUENCH EXPERIMENTS

Duplicate samples of ten selected dyes in vesicles of pure DOPC and of 85% DOPC and 15% of each of the spin-labeled lipids were investigated. Two-ml samples of the sonicated solutions were pipetted into cuvettes and placed in the cuvette holder of a Perkin Elmer LS 50 B fluorescence spectrometer. The samples were measured at excitation and emission slit widths of 5 nm respectively, except for 1QITC and 2QITC. These dyes showed very small fluorescence signals and were measured with both slits set to 7.5 nm. A 515-nm cut-off filter in the emission pathway was used for all measurements. The fluorescence was recorded in the excitation and emission maximum of each dye as given in Table 3.

Since an initial redistribution process of lipid and dye molecules between vesicles, aqueous buffer and cuvette walls took place after filling the vesicle suspension into a clean cuvette, the initial fluorescence decreased at the beginning of a 15-min long reading. This process could be represented by a mono- or bi-exponential decay and could be reproduced by the function $F(t) = \Delta F_1 \exp(-t/\tau_1) + \Delta F_2 \exp(-t/\tau_2) + F_{SS}$, where F is the detected fluorescence, t the time in seconds. ΔF_1 and ΔF_2 are the amplitudes of fluorescence decreases, τ_1 and τ_2 are the life times of this process. The second exponential was a (minor) instrumental artifact with $\tau_2 > 700$ sec and could be observed only in a few experiments. For comparison of the quenching effects of the doxyl labels on the styryl dyes the steady-state fluorescence level, F_{SS}, was used. The magnitude of quenching was determined as quench ratio, $Q = F_{SS}(+ \text{spin label})/F_{SS}(- \text{spin label})$, for each of the styryl dyes and quenchers.

Results

The original intention of the application of the synthesized fluorescent isothiocyanate dyes (Fig. 1) was to link them by their S=C=N-group covalently to an ion pump, as it has been performed with FITC (fluoresceine 5-isothiocyanate) before (Karlish, 1980), and to overcome by such a specific labeling the necessity of membrane preparations with high densities of pump proteins, which is an inevitable prerequisite for the investigations when the dye molecules are evenly distributed in the membrane, as has been explained in the Introduction. However, with all members of the nXITC dyes tested, independent of the length of the spacer between the polar isothiocyanate head group and the chromophore, it was impossible to obtain a (covalent) linkage between proteins and dyes. We propose that the main reason for this failure is the high partition coefficients of the dyes (Table 2), which led to a fast redistribution of the dye into the lipid phase of the membranes when both, dye and membranes, were
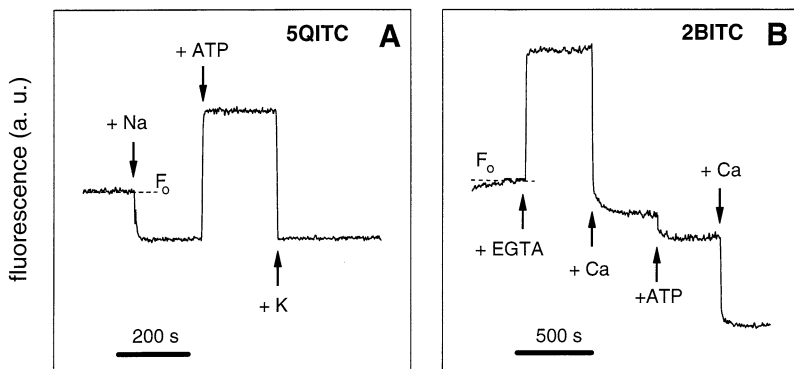
Fig. 2. Investigation of electrogenic partial reactions of the Na,K-ATPase and the SR Ca-ATPase with electrochromic styryl dyes by so-called standard experiments. (*A*) The electrochromic reaction steps that could be detected in the case of Na,K-ATPase and dye 5QITC. The detectable steps were cytoplasmic binding of Na^+ ions $(+ Na)$, $E_1 + 3Na_{cyt}^+ \rightarrow Na_3E_1$, when 50 mM NaCl were added to buffer containing neither Na^+ nor K^+ ions. Addition of 0.5 mM ATP induced the next sequence of steps $(+ ATP)$, $Na_3E_1 + ATP \rightarrow \ldots \rightarrow P\text{-}E_2 + 3Na_{ext}^+ + ADP$. The third step $(+ K)$ contains, after addition of 20 mM KCl, binding of K^+ ions to the $P\text{-}E_2$ state and the beginning of a continuous turnover, $P\text{-}E_2 + 2K_{ext}^+ \rightarrow E_2(K_2) + P_i \rightarrow \ldots$. The enzyme will remain preferentially in state $E_2(K_2)$ due to the following rate-limiting step. (*B*) In the case of the Ca-ATPase, the dye 2BITC was found to be an excellent detector of the electrochromic reaction steps. Starting with a buffer that contained sub-micromolar traces of Ca^{2+} ions, addition of 100 μM EGTA chelated all Ca^{2+} ions and led to state E_1. Addition of 190 μM $CaCl_2$ induced the transition into the state Ca_2E_1, addition of 1 mM ATP induced the transition preferentially into state $P\text{-}E_2$ and 15 mM $CaCl_2$ into state $P\text{-}E_2Ca_2$. This sequence of additions maintains successively the states Ca_xE_1, E_1 $(+ EGTA)$, Ca_2E_1 $(+ Ca)$, $P\text{-}E_2$ $(+ ATP)$ and $P\text{-}E_2Ca_2$ $(+ Ca)$.

mixed in an aqueous solution. A partition coefficient of $>10^4$ corresponds to the fact that more than 99.99% of the dye molecules are in the lipid phase. Under this condition the range and the degree of freedom of the isothiocyanate group were not high enough to find an appropriate lysine on the protein surface of the ion pumps to react with independently of the length of the spacer. However, even without the chemical linkage these electrochromic dyes exhibited a number of properties that make them useful for further applications, at least for experiments with membranes containing Na,K-ATPase and the SR Ca-ATPase and, perhaps, also with other ion pumps.

The fluorescence response of the *n*XITC dyes to an addition of negatively and positively charged hydrophobic ions, TPB^- and TPP^+, was in full agreement with the findings observed with RH421 earlier (Bühler et al., 1991). These experiments were performed with lipid vesicles, purified membrane preparations containing Na,K-ATPase, and Ca-ATPase-containing membranes from SR (*not shown*). Titration experiments with the Ca-ATPase in state E_1 with Ca^{2+}, Mg^{2+} and H^+ ions, demonstrated that the ion binding and release steps are detected by *n*XITC (Peinelt & Apell, 2002). This is also supported by experiments in which in the absence of Ca^{2+} and Mg^{2+} ions, when a considerable amount of the ion pumps is expected to be in state $E_2(H_2)$, ATP was added. The shift of the equilibrium from E_2 into state E_1 was not accompanied by a change in the fluorescence level (*not shown*).

DETERMINATION OF WAVELENGTHS FOR MAXIMAL RESPONSE ON SUBSTRATE-INDUCED PARTIAL REACTIONS

As has been shown previously with the styryl dye RH421, it is possible to maintain steady-state conditions of defined protein states by appropriate buffer conditions. In the case of the Na,K-ATPase a so-called standard experiment was introduced, which started in a buffer that initially contained 30 mM histidine, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.2, 200 nM fluorescent dye and 8–9 μg/ml protein. The initial state of the ion pumps obtained under this condition was E_1. The experiment was performed by consecutive addition of substrates to stabilize subsequent steady states in the pump cycle: $+$ 50 mM NaCl to reach the state Na_3E_1, $+$ 0.5 mM ATP transfers the protein mainly into state $P\text{-}E_2$, and $+$ 20 mM KCl maintains preferentially $E_2(K_2)$ (Stürmer et al., 1991). An example of such an experiment is shown in Fig. 2*A*. A correspondent standard experiment was also introduced for the SR Ca-ATPase (Butscher et al., 1999): in buffer initially containing 25 mM tricine, 50 mM KCl, 1 mM $MgCl_2$, and approximately 500 nM Ca^{2+} (according to Fura-2 measurements), pH 7.2, 200 nM fluorescent dye and protein (19 μg/ml). The initial state of the ion pump in this buffer condition was not well defined due to the undefined Ca^{2+} concentration, therefore, we named it Ca_xE_1 with approximately $x \leq 1$. The state E_1 was induced by addition of 0.1 mM EGTA, and subsequently Ca_2E_1 by adding 190 μM $CaCl_2$, $P\text{-}E_2$ by 1 mM ATP and $P\text{-}E_2Ca_2$ by 15 mM $CaCl_2$ (Fig. 2*B*).
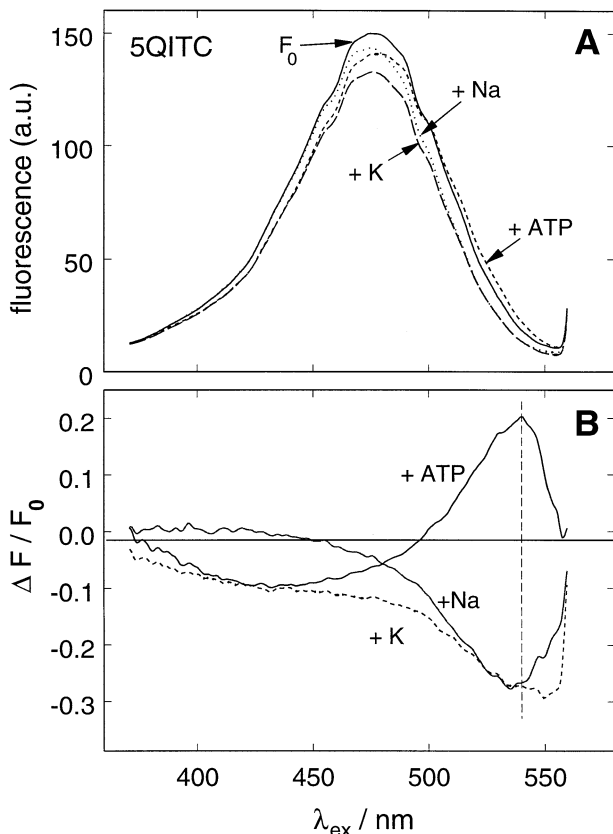
Fig. 3. Spectral dependence of the styryl dye 5QITC on different substrate conditions in membranes containing Na,K-ATPase. (*A*) In the presence of 200 nM 5QITC and 9 μg/ml Na,K-ATPase and in the absence of any monovalent cations in the buffer, the excitation spectrum was measured (solid line, marked F_0). The emission wavelength was set to 590 nm. Subsequently 50 mM NaCl (+ *Na*), 1 mM ATP (+ *ATP*) and 20 mM KCl (+ *K*) were added (*see* Fig. 2) and the respective spectra taken. The wavelength of the absorption maximum was 470 ± 5 nm. (*B*) The relative changes between the different spectra were calculated as $\Delta F/F_0 = (F_{+ \, substrate} - F_0)/F_0$ and plotted against the wavelength. The most significant fluorescence changes were found at the red edge of the excitation spectrum, $\lambda_{\Delta Fmax} = 540 \pm 5$ nm (dashed vertical line). The found maximum values of $\Delta F/F_0$ depend on the density of active Na,K-ATPase in the membranes and varied between experiments from different preparations.

At each steady-state condition of the standard experiments, emission spectra were taken for 19 of the styryl dyes introduced above. To increase the signal-to-noise ratio each spectrum was measured five times and the average calculated. An example is shown in Fig. 3*A*, in which 5QITC was applied in Na,K-ATPase-containing membranes. The excitation range was between 400 nm and 570 nm, the emission wavelength was kept constant at the emission maximum of 590 nm. To determine the excitation wavelength for optimal fluorescence changes upon substrate additions, normalized difference spectra were calculated between each substrate condition and the initial spectrum, F_0, which was taken before the

first substrate was added. The result is shown in Fig. 3*B*. From these experiments, for example, the optimum excitation wavelength of 5QITC was determined to be 542 nm, and in the experiment shown, the fluorescence levels of the different substrate conditions relative to the initial level, F_0, were -0.26 for state Na_3E_1, $+0.2$ for state P-E_2 and -0.27 for a mixture of states $E_2(K_2)$ and $(Na_3)E_1$-P. In Tables 3 and 4 the results are compiled, which were obtained as averages from a number of different enzyme preparations of Na,K-ATPase or SR Ca-ATPase with all dyes investigated. Control experiments with lipid vesicles made of DOPC by the dialysis method were performed to determine the effect of the substrates of the Na,K-ATPase, i.e., 50 mM NaCl, 0.5 mM ATP and 20 mM KCl, on the fluorescence of 5QITC when no ion pumps were present. An almost wavelength-independent decrease of the fluorescence, $\Delta F/F_0$, was observed as -2.5% (Na^+), -6% (ATP) and -9.3% (K^+), which can be discussed as the major source of the amplitude decrease of the absorption spectra visible in Fig. 3*A*.

EFFECT OF DYE CONCENTRATION

To investigate the effect of dye concentration on the amplitude of the fluorescence responses, standard experiments as shown in Fig. 2 were performed with 5QITC and Na,K-ATPase-containing membranes, as well as with 2BITC and SR membranes in a dye concentration range between 50 nM and 600 nM. The substrate-specific responses of both ion pumps, and, as derived quantity, the number of dye molecules per pump molecule, are shown as function of dye concentration in Fig. 4. To obtain maximum fluorescence responses at a minimum ratio of dye/pumps, for both protein preparations a concentration of 200 nM was chosen for most of the experiments. With the known ratio of protein/lipid of 1:1 (w/w) in the case of the Na,K-ATPase, about 175 lipid molecules per pump protein are estimated. In the presence of 200 nM dye, the lipid/dye ratio is 30.

ANALYSIS OF THE EFFECT OF THE LENGTH OF SPACER AND TAIL

The dependence of the substrate-induced fluorescence responses on the spacer length, s, and on the length of the alkyl chain, n, of the tail is shown in Fig. 5 for SR membranes containing Ca-ATPase and in Fig. 6 for Na,K-ATPase-containing membrane fragments.

When the resulting effects of the different dyes on both enzyme preparations are compared, the first striking observation is that in the case of the Na,K-ATPase a significant dependence on spacer and tail length was found, while in the case of the SR Ca-ATPase such a dependence could not be detected. From Fig. 5 it can be seen that variations of the re-

Table 3. Comparison of spectroscopic data obtained with membranes containing Na,K-ATPase and of substrate-specific responses (pH 7.2)

Dye	Excitation maximum λ/nm	Emission maximum λ/nm	Optimal detection at λ_{ex}/nm	$\Delta F_{Na}/F_0$	$\Delta F_{ATP}/F_0$	$\Delta F_K/F_0$
1PITC	488	578	0[a]	0[a]	0[a]	0[a]
2PITC	487	590	540.0	0.02	0.061	0.018
3PITC	483	583	542.0	−0.026	0.121	0.023
5PITC	470	590	542.0	−0.135	0.132	−0.095
2BITC	485	575	541.0	0.07	0.014	−0.037
3BITC	486	585	552.0	0	0.055	−0.01
4BITC	481	590	525.0	−0.02	0.083	0.023
5BITC	475	595	541.0	−0.092	0.129	−0.091
7BITC	482	587	541.5	0.041	0.414	0.139
1QITC	465	600	538.5	−0.067	0.069	−0.047
2QITC	470	595	539.5	−0.061	0.111	−0.087
3QITC	474	590	540.0	−0.065	0.108	−0.089
4QITC	470	595	542.5	−0.153	0.123	−0.141
5QITC	470	590	542.5	−0.280	0.249	−0.270
6QITC	470	585	540.5	−0.269	0.085	−0.228
7QITC	473	582	543.0	−0.126	0.116	−0.100
2HITC	475	590	a	0[a]	0[a]	0[a]
5HITC	470	595	540.0	−0.138	0.108	−0.180
6HITC	495	570	531.5	−0.130	0.129	−0.185

[a] 1PITC and 2HITC did not produce significant responses on substrate addition.

Table 4. Comparison of spectroscopic data obtained with SR membranes containing Ca-ATPase and of substrate specific responses (pH 7.2)

Dye	Excitation maximum λ/nm	Emission maximum λ/nm	Optimal detection at λ_{exc}/nm	$\Delta F_{EGTA}/F_0$	$\Delta F_{Ca}/F_0$	$\Delta F_{ATP}/F_0$
2PITC	481	590	525.0	0.505	0.002	−0.093
5PITC	483	576	546.5	0.422	0.098	0.121
2BITC	482	590	540.0	0.529	0.068	0.018
5BITC	483	584	546.0	0.344	0.008	0.025
7BITC	483	574	548.5	0.417	0.019	0.005
1QITC	470	585	537.5	0.420	0.129	0.137
2QITC	482	590	530.0	0.466	0.022	−0.022
3QITC	482	580	544.5	0.415	0.075	0.094
4QITC	482	583	543.0	0.476	0.084	0.022
5QITC	482	582	546.0	0.419	0.007	0.018
6QITC	482	570	540.0	0.382	0.06	0.093
7QITC	482	574	540.0	0.472	0.033	0.055
2HITC	481	590	530.0	0.518	−0.010	−0.038
5HITC	482	583	540.0	0.392	0.05	0.063

lative fluorescence level of state E_1 of the SR Ca-ATPase, which is maintained by addition of EGTA, are not significant with respect to the length of tail n or spacer s. The error bars of the data in traces b (state Ca_2E_1) and c (P-E_2) were removed for sake of clarity; they were of the same magnitude as those shown for trace a. There is a minor difference of the average of $\Delta F/F_0$ of state E_1 (trace a) between the 2XITC dyes (0.40 ± 0.02) and the 5XITC dyes (0.51 ± 0.01). When regression lines were fitted through the $\Delta F/F_0$ levels of the Ca_2E_1 and P-E_2 states, small slopes were obtained (*not shown*), however, there is no clear tendency, and horizontal lines with the am-plitude of the average values were within the range of the error bars (traces b and c). From a comparison of all investigated dyes we gave 2BITC the preference for experiments with SR Ca-ATPase.

In contrast to the findings with SR membranes containing Ca-ATPase, a clear dependence on tail or spacer length was found in the case of Na,K-ATPase-containing membrane preparations (Fig. 6). In Fig. 6A a pronounced response of $\Delta F/F_0$ was observed in the range of $n = 4$–6. It is more obvious for the states Na_3E_1 (trace a) and $E_2(K_2)$ (trace c) than for the state P-E_2 (trace b), where only 5QITC showed a $\Delta F/F_0$ greater than 0.2. When for the optimal tail length,
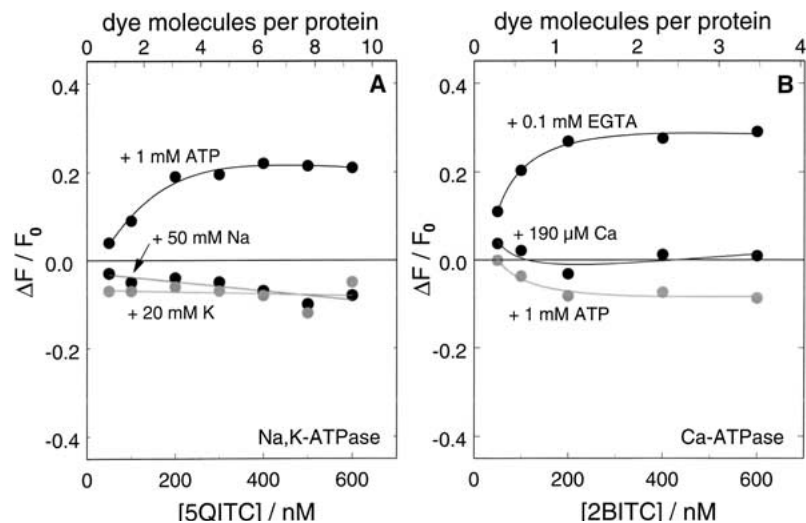
Fig. 4. Dependence of specific fluorescence response, $\Delta F/F_0$, on dye concentration. Standard experiments as shown in Fig. 2 were performed with Na,K-ATPase and 5QITC (A) and with Ca-ATPase and 2BITC (B) in a concentration range between 50 and 600 nM. With the known molar mass of the ion pumps, the amount of protein in the cuvette and the known high partition coefficients, the number of dye molecules per protein was estimated as indicated (upper x axes). The lowest dye concentration to get maximal $\Delta F/F_0$ was found to be 200 nM in both preparations.
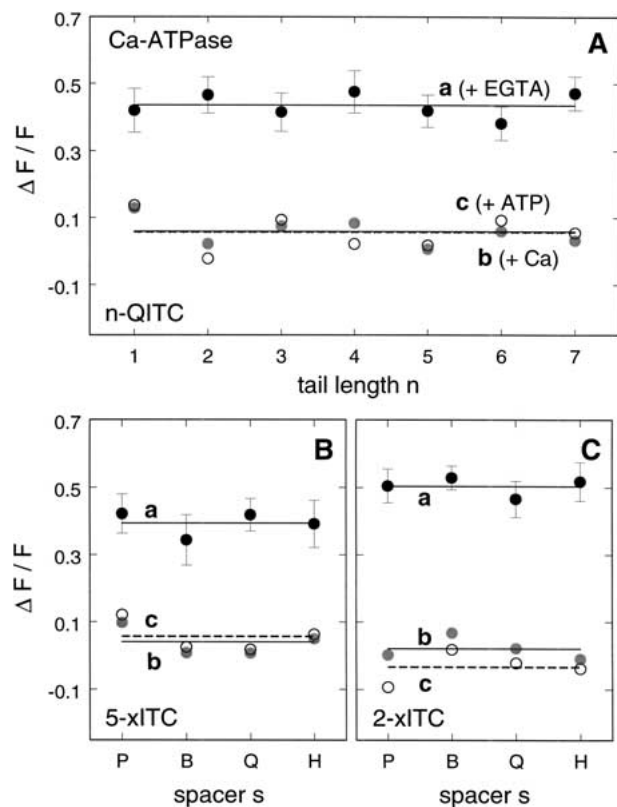
Fig. 5. Dependence of Ca-ATPase-specific fluorescence changes, $\Delta F/F_0$, on tail length, n, and spacer length, s, of the nXITC dyes. (A) With a spacer length of 5 CH$_2$ groups (QITC) the responses on addition of 0.1 mM EGTA (a), 190 μM CaCl$_2$ (b) and 1 mM ATP (c) are plotted against the number of carbon atoms in the alkyl chain. Results of the variations in the length of the spacer, s, are shown for $n = 5$ (B) and for $n = 2$ (C). Lines through the data represent the average value of the corresponding data set. The error bars for the data in traces b and c were removed for sake of clarity; they are of the same size as those shown for the data of trace a.

$n = 5$, the spacer s is varied (Fig. 6B), again only 5QITC showed significantly increased fluorescence

changes. Variation of s in dyes with a tail $n = 2$ revealed that these dyes could not detect the Na,K-ATPase-specific fluorescence by significantly large fluorescence changes (Fig. 6C). Comparison of all tested nXITC dyes showed that 5QITC is the most appropriate dye with which to investigate electrogenic partial reactions of the Na,K-ATPase.

When 5QITC was compared with the well established styryl dye RH421, it turned out they are comparable, although the response on the ATP-induced partial reaction, $Na_3E_1 \rightarrow ... \rightarrow P\text{-}E_2$, is more pronounced in the case of RH421 (Table 5). In contrast to RH421, with which the states Na_3E_1 and $E_2(K_2)$ had different fluorescence intensities, in the case of 5QITC, both states had the same levels. However, most of the apparent fluorescence decrease in state $E_2(K_2)$ has to be accounted to the unspecific effect observed also in pure lipid vesicles (see above). In the case of the SR Ca-ATPase only minor differences of $\Delta F/F_0$ between the different nXITC dyes were found, however, RH421 has been shown to produce no significant changes for any of the electrogenic partial reactions of the Ca-ATPase (Butscher et al., 1999).

ESTIMATION OF THE POSITION OF THE STYRYL CHROMOPHORES IN THE LIPID PHASE OF THE MEMBRANE

The observed dependence of $\Delta F/F_0$ of the different Na,K-ATPase states on the nXITC dyes led to the idea to determine the position of the chromophore vertical to the surface of the membrane. A tool available to get information on the vertical position was the use of doxyl-labeled lipids with defined position of the quenching group in the lipid bilayer. Doxyl labels attached to lipid molecules may be used as effective quenchers of the fluorescence of membrane-bound (styryl) dyes (Chattopadhyay & Lon-
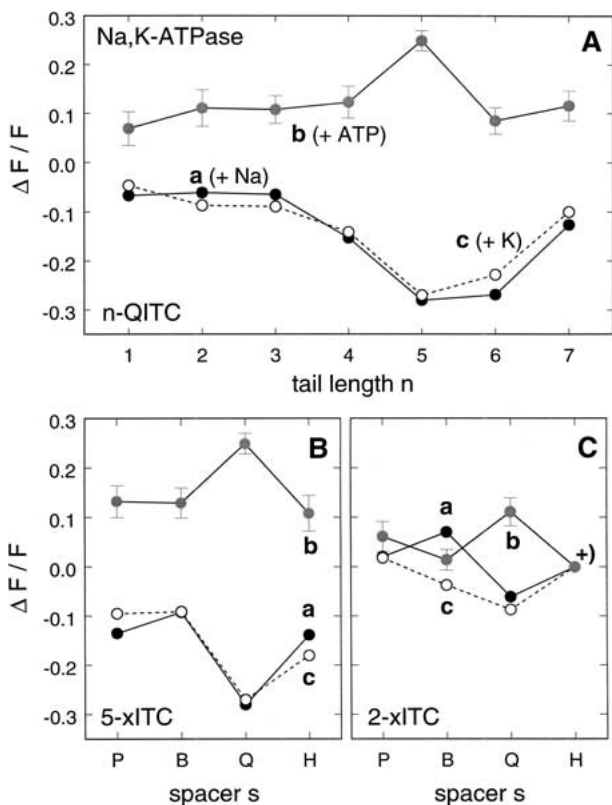
Table 5. Comparison of 5QITC and RH421

Partial reaction into state	$\Delta F / F_0$	
	5QITC	RH421
Na_3E_1	−0.28	−0.25
P-E_2	+0.25	+0.37
$E_2(K_2)$	−0.27	−0.15

5QITC and RH421 were compared with respect to their response on electrogenic partial reactions of the Na,K-ATPase from the so-called standard experiment. The numbers given are averages from numerous experiments.
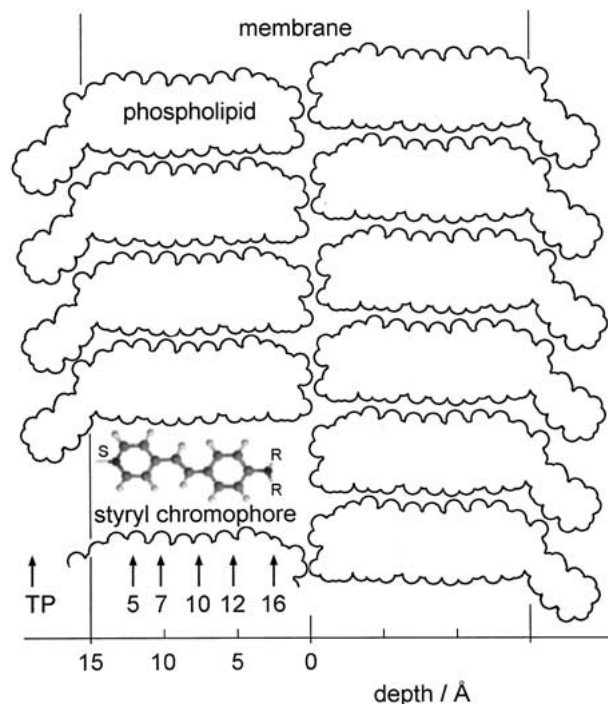
Fig. 6. Dependence of Na,K-ATPase-specific fluorescence changes, $\Delta F/F_0$, on tail length, n, and spacer length, s, of the nXITC dyes. (*A*) With a spacer length of 5 CH_2 groups (QITC), the responses of addition of 50 mM NaCl (*a*), 1 mM ATP (*b*) and 20 mM KCl (*c*) are plotted against the number of carbon atoms in the alkyl chain. Results of the variations in the length of the spacer, *s*, are shown for $n = 5$ (*B*) and for $n = 2$ (*C*). It can be seen that the different spacers for $n = 2$ do not significantly alter the small responses. In the case of $n = 5$, the dyes 5PITC and 5BITC produced only half of the fluorescence changes (or less) when compared with 5QITC. With 2XITC no significant responses could be obtained. Lines connecting the data are drawn to guide the eye. The error bars for the data in traces *b* and *c* were removed for sake of clarity; they are of the same size as of those shown for the data of trace *a*.

Fig. 7. Visualization of size and (possible) position of the nXITC chromophore in a lipid bilayer. The arrows indicate the position of the doxyl labels used, which served as quenchers of the nXITC fluorescence. The numbers indicate the number of the carbon atom of the acyl chain to which the doxyl label is attached. TP: TEMPO PC.

don, 1987; Kachel, Asuncion-Punzalan & London, 1998; Kaiser & London, 1998). Since the quenching group can be attached to different carbons of the acyl chain of the lipid molecule, they are located at different distances from the membrane surface, and their efficacy to quench the fluorescence could provide information on their distance to (the sensitive part of) the chromophore. From Fig. 7, however, it is obvious that the large size of the chromophore will confine the spatial resolution of its position. The arrows in Fig. 7 indicate the position of the doxyl groups in the respective lipid molecule.

It is not possible to import lipids with a quenching spin label in a defined concentration into existing, protein-containing membrane preparations. Therefore, experiments to collect information on the position of the styryl chromophore were performed with vesicle preparations whose ratio of dye/quencher/lipid could be set deliberately and controlled, as described in Materials and Methods. In the experiments performed the concentrations were chosen to be 1 dye molecule per 45 doxyl-labeled and 255 unmodified PC molecules. The total lipid concentration was 60 μM. The steady-state fluorescence of the quenched and unquenched vesicle preparations was measured as shown in Fig. 8A. In these experiments it was observed that the fluorescence decreased initially in an exponential way with time constants in the order of 200 sec. The decrease probably reflected adsorption to or spreading of lipid and dye molecules on the surfaces of the aqueous phase. The data could
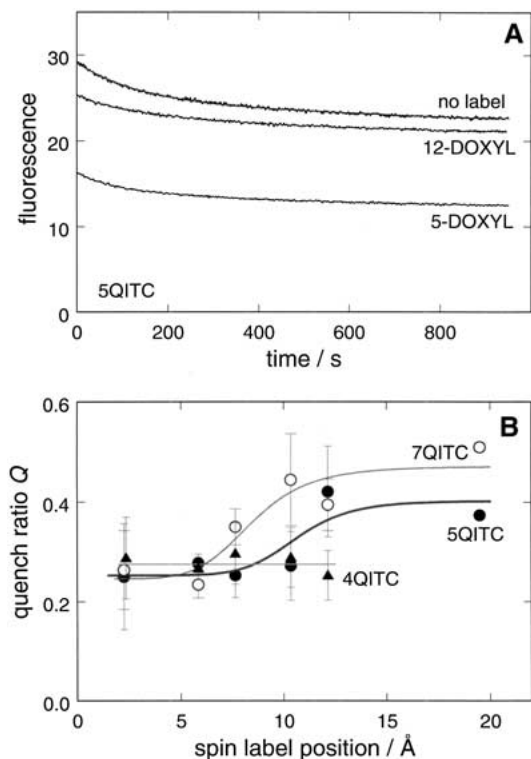
Fig. 8. Quenching of nXITC dyes by doxyl labels attached to various carbons of the fatty acid chains. (*A*) Time course of the fluorescence intensity when freshly prepared vesicles consisting of 5QITC/doxyl lipid/DOPC (1:45:255) were filled into a cuvette. The unquenched fluorescence is compared with preparations containing 12-doxyl-and 5-doxy-labeled lipid. The steady-state level was determined using a fit function that reproduced the initial effects by two decaying exponential functions (fitting line through the "no label" data). (*B*) The quench effect of the different doxyl labels on three nQITC dyes. The quench ratio Q (*see* text) was plotted against the position where the quencher is proposed to be situated in the membrane (Fig. 7). The lines were drawn to visualize the position dependence of quench effects in the case of 4QITC (triangles), 5QITC (filled circles) and 7QITC (open circles). The error bars represent the standard deviation of experiments performed under identical conditions.

Fig. 9. Average quench ratio, Q_{Av}, of different nXITC dyes. (*A*) Solid circles represent the dependence of Q_{AV} on tail length for $n = 1$ to 4. The error bars are standard deviations calculated from the experiments with all doxyl labels and with TEMPO PC (if measured). In the case of $n \geq 4$ a significant dependence of the spin label position was observed. The solid circles represent the quench ratio obtained from the inner quenchers, the open circles that of quenchers close to the membrane surface (*see* Fig. 8). (*B*) Dependence on spacer length of the 5XITC dyes. The solid circles represent the averaged quench ratio of the experiments with doxyl labels, the open circles are from measurements with TEMPO PC. In the case of 5PITC, only TEMPO PC produced a significant quench effect.

be fitted perfectly with the function given in Methods as exemplified for the uppermost fluorescence trace in Fig. 8*A*. For each of the investigated dyes the steady-state fluorescence obtained in the presence of one of the five quenching doxyl labels (or TEMPO PC) was compared to the fluorescence level in the absence of a quencher by calculating the quench ratio, $Q = F_{+quencher}/F_{-quencher}$. Part of the results are shown in Fig. 8*B*, where Q is plotted against the position of the doxyl label within the lipid bilayer for three of the dyes. The positions were calculated according to a published estimate (Sassaroli et al., 1995).

   It was found that in the case of short alkyl chains, $n < 4$, the quench ratio, Q, was not significantly dependent on the position of the quencher and, therefore, an average value of Q could be calculated. A tendency that quenching is stronger when
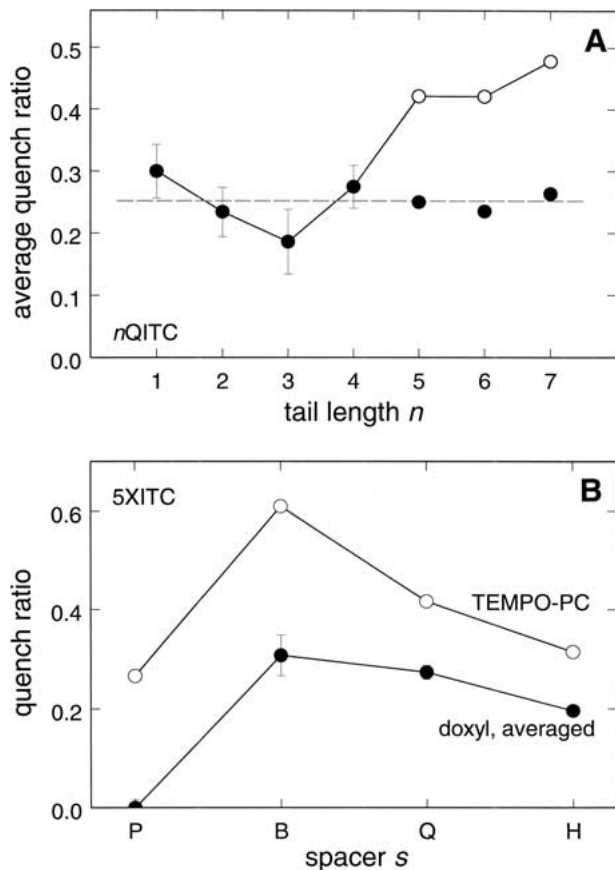
the position of the doxyl label is closer to the surface of the membrane, was found in the case of the dyes with the longer alkyl chains, 5QITC to 7QITC (Fig. 8*B*). The large error bars of most of the data points reflect the poor reproducibility of this type of experiments. In consequence, we determined an average quench ratio, Q_{AV}, obtained from all 5 doxyl labels (and from TEMPO-PC, if measured) in the case of 1QITC to 4QITC. In the case of 5QITC to 7QITC we made a distinction between the Q of the inner and of the outer quenchers. The results are shown in Fig. 9*A*. It is obvious that the effect of spacer, s, had a greater impact on the quench ratio, Q_{AV}, than the length, n, of the alkyl chains of the tail (Fig. 9*B*). The dyes with the shortest spacer, $s = 3$ (nPITC), were hardly affected by the intra-membrane quenchers ($Q_{AV} = 0.0$

$\pm$ 0.016). Only in vesicles with TEMPO-PC, which has the quenching label attached to the polar head group, a quench ratio $Q = 0.26 \pm 0.02$ was detected. That was interpreted as indication that the dye with the shortest spacer probably did not insert into the hydrophobic core of the membrane but remained with the sensitive part of its chromophore close to the polar head groups where the TEMPO label is located. BITC and QITC dyes ($s = 4$ and 5, respectively) were quenched by about 28 ± 2 % by the doxyl labels and 50 ± 10 % by TEMPO-PC. An even longer spacer, HITC ($s = 6$), again, had a smaller quench ratio (Fig. 9B).

These findings are in agreement with the concept that the depth of the styryl chromophore is shifted within the lipid membrane and that this is a function of the spacer length. The dependence of Q as function of the hydrophobic tails was less pronounced (Fig. 9A). Most of the averaged Q values for different tail length, n, did not significantly deviate from the overall average determined from all QITC dyes of 0.249 ± 0.014. Only the dye 3QITC was less strongly quenched when compared with the average (0.186 ± 0.052). As shown in Fig. 8B, in the case of 5QITC and 7QITC, and also in 6QITC (*not shown*), the quench ratio, Q, was in the order of 0.25 for spin labels with a position deeper inside the membrane ($x \leq 10$ Å), which is about the average quench ratio (Fig. 9A), but it was significantly larger for $x > 10$ Å ($Q \approx 0.4$–0.5).

Discussion

Position of the Chromophore of the nXITC Dye

In the literature, the fluorescence responses of various styryl dyes on the same reaction steps of the Na,K-ATPase gave a lot of discussion on the detection mechanism of these probes. Especially the contribution of the effect of a specific interaction of dye molecule with changing protein conformations (Fedosova et al., 1995) or the electrochromic effect of a position-dependent sensitivity for charge movements (Bühler et al., 1991) were discussed as origin of the fluorescence changes. When originally tested in nerve membranes, typical fluorescence responses, $\Delta F/F_0$, of styryl dyes on changes of the transmembrane voltage were about 14 to 20% per 100 mV (Grinvald et al., 1987). In the case of open membrane fragments containing Na,K-ATPase (where both sides of the membrane are short-circuited and no transmembrane potential is possible) certain partial reactions are accompanied by relative fluorescence changes with amplitudes in the order of 100% (Klodos & Forbush, H., 1988, Bühler et al., 1991).

Now a more systematic analysis is possible with a family of styryl dyes, called nXITC, which have the same chromophore but in which the length of the spacer, s, between the chromophore and the polar (but uncharged) head group and the length of the two (identical) hydrophobic tails is systematically varied.

In the case of the Na,K-ATPase, a dependence on spacer and tail length of the nXITC dyes was found: the addition of a single CH_2-group to spacer or tail could already produce significant differences in the substrate-dependent fluorescence changes (Fig. 6). Therefore, it was important to study properties of these dyes systematically to gain information on the origin of the observed behavior and how these effects depended on the chromophore position vertically to the plane of the membrane in the lipid bilayer.

Systematic and quantitative experiments on the position dependence had to be performed with artificial lipid vesicles, which allowed the precise control of concentrations of lipid, dye and quenching agents that was necessary to compare different experiments. This technique was applied successfully before (Chattopadhyay & London, 1987, Kachel et al., 1998, Kaiser & London, 1998).

A method introduced by Chattopadhyay and London (1987) allows the determination of several parameters concerning the distances between a chromophoric group within the lipid phase and quencher molecules attached to the acyl chain of lipids. One parameter is the critical radius, R_C, which defines the sphere around the spin label within which quenching occurs. From the experiments performed in the framework of this paper, an average value of $R_C = 5.56 \pm 0.87$ Å could be determined for the interaction of doxyl quenchers and nXITC dyes. This number has to be compared with the size of the chromophore (Fig. 7) and it provides an idea on the spatial resolution which has to be taken into account when the position of the nXITC chromophore will be discussed.

Assuming that in the excited state of the chromophore a delocalized electron is shifted from the inner aniline unit of the chromophore to the pyridine unit closer to the membrane surface (Fischer, 1974), then the quench effect of the excited state is to be expected preferentially in proximity to the pyridine unit of the chromophore. (Charge shifts up to 3 nm were reported between ground and excited state of styryl dyes (Fromherz & Lambacher, 1991).) From the results shown in Fig. 8 and 9 it can be seen that the doxyl-labeled lipids are able to quench all scrutinized nXITC dyes (with exception of 5PITC) in pure lipid vesicles, with an average quench ratio of about 0.25 when the ratio labeled lipid to total lipid was 0.15. The maximum quench effect in the order of 0.4 to 0.5 was observed with the doxyl labels, which were positioned more closely to the membrane surface (Fig. 8), and with TEMPO PC, which has the quenching label in the region of the polar lipid head

group. TEMPO PC had always a significantly stronger effect than the quenchers attached to the acyl chain of the lipid (Fig. 9). Obviously, the position dependence of the quench effect was too unspecific to be able to pin down a defined position of the chromophore in the nXITC dyes tested. However, from Fig. 8B and 9A it can be seen that in the series of nQITC dyes all members with a tail of $n \leq 4$ were affected in the same way by each of 5 doxyl quenchers, while in the case of $n \geq 5$, an increase of the quench ratio, Q, could be observed when the doxyl labels had a position further than 10 Å away from the middle of the membrane, and in the case of $n = 7$, an increase of Q was observed at about 7.5 Å.

The absence of any position dependence of the quench effect in the case of nXITC dyes with hydrophobic tails shorter than 5 carbon atoms ($n \leq 4$) may be explained by either a broad distribution of the dye molecules perpendicular to the membrane plane within the hydrophobic core of the membrane, so that all doxyl labels had an equal share of chromophores within the critical radius, R_C, or, more probable, by the fact that the aniline unit of the dye was placed, independent of the respective dye, at a distance of about 10 Å from the center of the membrane, so that the delocalized excited electron was within R_C of all doxyl labels. When the alkyl chains of the dyes become longer, then they may preferentially not inter-digitate with the acyl chains of the opposite lipid leaflet, but push the chromophore towards the membrane surface and increase the quench effect, Q, of the doxyl labels at the positions $x \geq 10$ Å from the middle of the membrane. These assumptions would explain the observed dependence of the quench ratio, Q, on the tail length of the nXITC dyes.

The effect of the spacer, s, on Q is shown in Fig. 9B. Eye-catching is the difference of the efficacy to quench between TEMPO-PC and the doxyl labels, which was almost a factor of 2. This indicates that the electron in the excited state of the dyes has to be closer to the lipid head groups, where the TEMPO label is attached, than to the acyl chains, which carry the doxyl labels. A special situation was found in the case of the 5PITC dye, which was quenched only by the TEMPO label and not significantly by any of the doxyl labels. A possible explanation for this observation would be that the dye did not insert properly into the hydrophobic matrix of the pure DOPC lipid bilayer. In the case of Na,K-ATPase-containing membranes with numerous different lipids, the series of nPITC dyes showed small or no significant response on the electrogenic partial reactions (Table 3), while in contrast in the SR membrane vesicles with the Ca-ATPase, significant fluorescence changes could be detected (Table 4). So far no explanation can be provided for these observations.

On the basis of the presented experimental results, the spatial resolution of the position of the chromophore is very poor and, unfortunately, the different members of the nXITC dye family may not be used to probe the strength of local electric fields at defined positions within lipid bilayers (or protein-containing membranes). However, in the case of the nQITC series, it was found that 5QITC to 7QITC were significantly more strongly affected by quenchers located in the range between 10 nm and the surface of the membrane (Figs. 8 and 9), a finding which is in agreement with the concept that the pyridine unit is positioned between 10 nm and 15 nm from the center of the bilayer.

DETECTION MECHANISM OF THE STYRYL DYES

To discuss the question of the mechanism of major contribution to fluorescence changes induced by partial reactions of the P-type ion pumps, a number of observations shall be compiled that are supporting a preferentially electrochromic detection mechanism of styryl dyes, such as RH421 and the nXITC: (1) It is known that fluorescence changes, as they are observed by partial reactions of the Na,K-ATPase pump cycle, can be induced by incorporation of hydrophobic ions, such as TPB^- and TPP^+, and the increase or decrease of the fluorescence intensity is always and in the same way correlated with the sign of the charges (Bühler et al., 1991). This was also found in the case of the nXITC dyes. (2) In Fig. 4 the concentration dependence of the fluorescence signals shows that an increase of the dye concentration from 200 nM to 600 nM did not significantly alter the fluorescence change, $\Delta F/F_0$, of the investigated reaction steps of the ion pumps. This concentration independence may be better explained by effects of local electric fields than by a (specific) interaction of dye molecules with altering protein conformations. In the latter case, 9 dye molecules would have to interact with the protein in the same way as 3 molecules do (Fig. 4A). If a considerable fraction of dye molecules would not participate in the detection process of the protein conformation, then $\Delta F/F_0$ would have to decrease in a hyperbolic concentration dependence when the "interaction sites" of the protein are occupied by dye molecules. If, in contrast, the dye molecules are diffusing through the lipid phase of the membrane, an increase of the dye concentration would hardly affect the amplitude of $\Delta F/F_0$ because the (average) electric field in the membrane is not significantly altered when the ratio of 60 lipids per dye molecule is decreased to 20. (3) All significant fluorescence changes cohere with partial reactions of the ion pumps in which net charges are imported into or released from the protein (Stürmer et al., 1991; Stürmer & Apell, 1992; Heyse et al., 1994; Bühler & Apell, 1995; Domaszewicz & Apell, 1999; Schneeberger & Apell, 1999). On the other hand, it was shown that partial reactions, which contain con-

formational transitions but no change of the number of ions inside the protein, did not affect the fluorescence emission, such as $E_1 + 2K^+ \rightarrow E_2(K_2)$ (Stürmer et al., 1991; Bühler & Apell, 1995; Schneeberger & Apell, 2001) or $Na_3E_1 \rightarrow P\text{-}E_2Na_3$, which can be observed in high Na^+ concentrations (Stürmer & Apell, 1992; Heyse et al., 1994). In the case of 2BITC and 2HITC, conformational transitions of the SR Ca-ATPase were also not accompanied by significant fluorescence changes (Butscher et al., 1999, Peinelt & Apell, 2002). (4) The comparison of direct electric measurements and fluorescence experiments with RH421 demonstrated that the Na^+-induced effects on the cytoplasmic side of the Na,K-ATPase coincide perfectly (Domaszewicz & Apell, 1999). I.e., RH421 detects accurately the electrogenic binding and release of Na^+. Therefore, we think that it is justified to analyze and interpret the results of the experiments on pump functions in this paper on the basis of ion movements detected by the electric field sensitivity of the nXITC dyes.

DETECTION OF PUMP FUNCTION BY nXITC

A number of dyes of the nXITC dye family could be used in a corresponding fashion to RH421 to detect substrate-induced partial reactions of the Na,K-ATPase (Table 3). The so-called standard experiments (Fig. 2) had basically the same appearance for RH421 and the nXITC dyes, only the amplitudes varied as function of header and tail length (Table 3, Fig. 6). With respect to the specific fluorescence changes, $\Delta F/F_0$, the dye optimal for Na,K-ATPase experiments, 5QITC, behaved comparable to RH421 (Table 5). In the case of the SR Ca-ATPase, the well established RH421 did not produce significant fluorescence changes (Butscher et al., 1999) but most of the nXITC did (Table 4, Fig. 5).

When the spectral dependence of the 5QITC fluorescence on states of the pump cycle was investigated (Fig. 3), not only a red or blue shift according the electrochromic effect was found, but in addition, changes in the fluorescence yield. When the spectra were normalized to the same fluorescence maximum, it could be seen that distinct shifts of the absorption spectra exist between F_0 and $F(+Na)$ and $F(+ATP)$; however, the shift between F_0 and $F(+K)$ is very small (<1 nm, *not shown*). Control experiments with lipid vesicles without protein revealed that addition of Na^+, ATP and K^+ produced a reduction of the fluorescence yield of 5QITC over the whole absorption spectrum with similar reductions at the maximum as they were observed with the Na,K-ATPase-containing membrane fragments, however, without a shift of the maximum wavelength (*not shown*). A possible explanation for the observed fluorescence decrease may be that the smaller chromophore of the nXITC dyes (when compared with

that of RH421) is closer to the membrane surface, and therefore more strongly affected by the addition of ionic substrates to the buffer. The pronounced effect of K^+ may be caused by the fact that the membrane is tenfold more permeable to K^+ than to Na^+ ions, which is mainly an effect of a higher partition coefficient of K^+, and that K^+ ions might preferentially accumulate in the superficial part of the membrane.

In the case of the Ca-ATPase, none of the nXITC dyes investigated showed a significant dependence on spacer or tail length for any of the tested partial reactions of the ion pump (Fig. 5). As has been shown recently (Butscher et al., 1999), ion-binding steps on both sides of the membrane produced large fluorescence changes (Fig. 2B), while the conformational transition, $Ca_2E_1 \rightarrow P\text{-}E_2$ was accompanied only by minor fluorescence changes below 10% (depending on buffer pH). The observed absence of any significant dependence of the fluorescence responses on dye structure may be explained on the basis of the publication of a crystal structure of SR Ca-ATPase with a resolution of 2.6 Å (Toyoshima et al., 2000). According to the structural details that became available by this analysis, the 2 Ca^{2+} ions are located side by side, with a distance of 5.7 Å, right in the middle of the transmembrane section of the protein. Under such a condition the potential-sensitive chromophores of all nXITC dyes are placed between the aqueous phase and the position of the charges in the ion pump that produce the (symmetric) electric potential within the membrane dielectric. If the dielectric constant of the hydrophobic membrane interior is not very inhomogeneous, then the chromophores of all nXITC dyes will detect approximately the same strength of the electric field and, in consequence, report the same fluorescence changes when ions are bound or released, as it was observed in the experiments presented (Fig. 5).

In the case of the Na, K-ATPase, an explicit dependence on tail and spacer length of the nXITC dyes was found (Fig. 6). The maximum fluorescence changes of substrate-specific responses were detected with 5QITC. The addition or removal of one CH_2 group from the spacer between the polar head group and the chromophore reduced the fluorescence responses by more than a factor of 2 (Fig. 6B). A similarly pronounced dependence on the number of CH_2 groups was found for the ATP-dependent partial reaction when the tail length of the QITC dyes was altered (trace *b* in Fig. 6A). In contrast, the ion binding and release reactions were affected in a broader range of tail lengths (traces *a, c* in Fig. 6A). On the basis of the interpretation of position-dependent quench of the nQITC (Figs. 8 and 9) it can be concluded qualitatively that the position of the electric field-producing charges have to be placed differently within the Na,K-ATPase in the states Na_3E_1,

P-E_2 and $E_2(K_2)$, which were characterized by the fluorescence levels plotted in Fig. 6. Unfortunately, so far no structural details of ion-binding sites of the Na,K-ATPase are available with a spatial resolution comparable to that of the Ca-ATPase. However, in a recent publication it has been shown that both, direct electric measurements and fluorescence experiments with the styryl dye RH421, prove that binding of the third Na$^+$ ion on the cytoplasmic side, Na$_2E_1 \rightarrow$ Na$_3E_1$, is an electrogenic process, and that the location of the bound Na$^+$ is 22–25% within the membrane dielectric (Domaszewicz & Apell, 1999). This number refers to a *dielectric* distance, which may differ from the spatial distance (dependent on constancy or variation of the dielectric coefficient of the membrane interior). The quench ratios, Q, in the series of 5QITC to 7QITC showed the greatest changes between the spin labels placed at a distance of below 7 Å and beyond 10 Å from the center of the membrane (Fig. 8*B*). Therefore, the proposal is suggestive to place the outer pyridine unit, which carries the delocalized electron in the excited state of the dye, in the range between 7 Å from the center of the membrane and its outside. To obtain a maximum fluorescence response on Na$^+$ binding, as in the case of 5QITC and 6QITC, the corresponding binding site for the 'electrogenic' Na$^+$ ion would be expected at a position between the aniline and pyridine unit, since then the bound Na$^+$ would introduce the maximum change of the local electric field for the electron when it moves into the excited state of the chromophore. On the basis of such a speculation, the location of the Na$^+$ binding site has to be situated at a topographic depth of ~20 % from the membrane surface (Fig. 7), a distance which is in a fair agreement with the dielectric determination.

STRUCTURE-FUNCTION RELATIONS DEDUCED FROM THE EXPERIMENTAL OBSERVATIONS

Even if the determination of the position of the Na$^+$ binding site at the moment has a number of speculative components, the striking difference between the SR Ca-ATPase and the Na,K-ATPase with respect to their effect on the fluorescence changes of the nXITC, dyes, is very clear. This indicates that with respect to ion binding and the position of the binding sites inside the proteins significant differences exist, despite the fact that both pumps are close relatives in the family of P-type ATPases and that it was possible to construct chimeras between both ion pumps, for which at least enzymatic activity could be established (Luckie et al., 1991, 1992; Ishii et al., 1994). The results of the crystal structure of the SR Ca-ATPase (Toyoshima et al., 2000), which revealed the position of the Ca^{2+} binding sites in the middle of the membrane, and of the electrogenicity of all ion-binding steps, Ca^{2+} and H$^+$ ions (Butscher et al., 1999, Pei-

nelt & Apell, 2002), as well as the similar fluorescence responses of all nXITC dyes on the investigated partial reactions as shown in this paper, fit well together. This structural concept of ion-binding sites that are located in the center of the membrane, is in strong contrast to the observations made with the Na,K-ATPase so far: On the cytoplasmic side, binding of only the third Na$^+$ ion is electrogenic, i.e., within the protein dielectric; all other ions, be that Na$^+$ or K$^+$, have to bind close to the surface or in wide, water-filled vestibules that are still part of the dielectric protein surface (Goldshlegger et al., 1987, Bahinski et al., 1988, Rakowski et al., 1990, Domaszewicz & Apell, 1999, Schneeberger & Apell, 2001). In the experiments presented it could be shown unambiguously that various dyes respond differently on electrogenic Na$^+$ binding, which is a strong indication that an ion is being bound within the sensitive length of the styryl chromophores and not in the center of the membrane. This finding proposes a significant difference of structure and placement of the ion binding sites in the Na,K-ATPase and the Ca-ATPase.

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