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Membrane Chloride Transport Measured Using a Chloride-Sensitive Fluorescent Probe†

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ABSTRACT: Transport of chloride across cell membranes through exchange, cotransport, or conductive pathways is a subject of great biological importance. Current methods of measurement are restricted in their sensitivity, time resolution, and applicability. A new transport measurement technique has been developed on the basis of the fluorescence quenching by chloride of the dye 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ). SPQ fluorescence quenching by chloride is rapid (<1 ms) and sensitive, with a greater than 50% decrease in fluorescence at 10 mM chloride. SPQ fluorescence is not altered by other physiological anions or by pH and can be used to measure both neutral and conductive transport processes. The high water solubility and membrane permeability properties of SPQ make it ideal for use in both membrane vesicles and cells. Chloride transport determined with SPQ was validated by measurement of erythrocyte chloride/anion exchange and membrane vesicle chloride conductance.

Membrane transport of chloride has an important role in cellular regulatory, absorptive, and secretory processes. Mechanisms of chloride transport include neutral chloride–anion exchange, neutral chloride–cation cotransport, and conductive chloride transport (Shennan et al., 1986; Geck & Heinz, 1986; Cupoletti & Sachs, 1984). The techniques currently used for measurement of chloride transport are patch-clamp, microelectrode, and ^{36}Cl tracer methods. Each method has specific applications but significant restrictions. Patch-clamp techniques apply only to conductive transport in isolated cells or membrane fragments and cannot be applied easily to vesicles or to intact tissues. Microelectrodes are invasive, lack sensitivity and specificity, and show poor response rates for measurements of non-steady-state chloride transients. Tracer methods are insensitive because of the low specific activity of ^{36}Cl. They require relatively large sample volumes and cannot be used for determination of transport processes that exhibit rapid response rates or require high time resolution.

We describe here the development of a noninvasive method for the measurement of membrane chloride transport that is sensitive, has a rapid response time, and can be used in membrane vesicles and cells. It is based on the quenching of a water-soluble fluorescent dye [6-methoxy-*N*-(3-sulfopropyl)quinolinium, SPQ; Figure 1] by chloride. The synthesis of SPQ and the halide sensitivity of its fluorescence were reported by Wolfbeis and Urbano (1983); however, this probe has not been used for measurement of chloride in biological samples. On the basis of studies in biomembrane systems, we find that SPQ can be loaded into membrane vesicles and cells, where it reports accurately the instantaneous chloride concentration.

Materials and Methods

6-Methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ; Figure 1) was synthesized by the method of Wolfbeis and Urbano (1982). H_2DIDS (dihydro-4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) was obtained from Molecular Probes (Junction City, OR), SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) and DPC (diphenylamine-2-carboxylate) were obtained from ICN Biomedicals Inc. (Plainview, NY), and $H_2{}^{35}SO_4$ was obtained from New England Nuclear (Boston, MA).

SPQ fluorescence was excited at 350 nm (8-nm band-pass) and measured with a GG420 Schott glass cut-on filter (Melles-Griot, Irvine, CA) in an SLM 4800 spectrofluorometer (Urbana, IL) interfaced to an IBM PC/XT computer. Samples were contained in acrylic cuvettes stirred continuously in a thermostated cuvette holder. SPQ fluorescence intensity was averaged over 1-s time intervals during time course measurements. Measurements of fluorescence lifetimes were carried out by phase-modulation fluorometry. Excitation light was modulated at 6, 18, and 30 MHz, and phase and modulation lifetimes were measured relative to a reference standard, dimethyl-POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazolyl)-benzene] in ethanol (τ = 1.45 ns at 23 °C; Lakowicz et al., 1981). A KV408 cut-on filter (Schott Optical Glass Inc., Duryea, PA) was placed in front of the GG420 filter for fluorescence lifetime measurements. Ground-state heterogeneity analysis was performed with three-frequency phase and modulation data as described previously (Illsley et al., 1987b).

Stopped-flow experiments were carried out on a Dionex 130 stopped-flow apparatus (Sunnyvale, CA). SPQ fluorescence was excited at 360 nm (16-nm band-pass) with a Zeiss double monochromator and measured using a 420-nm cut-on filter. Solutions containing SPQ (in solution or trapped in cells) were exposed to chloride gradients, and the time course of SPQ fluorescence was recorded at a maximum acquisition rate of 40 μs/point.

Sealed human erythrocyte ghosts were prepared by the method of Steck and Kant (1974) and maintained at 4 °C prior to use. Ghosts were loaded with SPQ by incubation for 16 h at 4 °C in 100 mM sucrose, 100 mM KCl, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–tris(hydroxymethyl)aminomethane (HEPES–Tris), pH 7.00, containing 1 mM SPQ. Ghosts were also loaded with SPQ by

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incubation with 10 mM SPQ for 30 min at 37 °C. Placental microvillous vesicles (MVV) were prepared by the method of Illsley and Verkman (1986b) and stored at −70 °C. MVV were loaded with SPQ by incubation with 10 mM SPQ for 12–24 h at 4 °C. Cells and vesicles were washed twice in the incubation buffer (minus SPQ) immediately prior to use and maintained at 4 °C. Leakage of SPQ at 4 °C, as assessed by measurement of the intravesicular or intracellular fluorescence signal, was less than 5%/h.

In all experiments the intracellular or intravesicular osmotic and ionic strengths were balanced with those in the external medium. Where noted, valinomycin was added from ethanolic stock (25 mg/mL) to give a concentration of 10 μg/mg of membrane protein. The valinomycin concentration used (10 μg/mg of membrane protein) was determined from experiments in which the chloride influx rate was measured at a series of valinomycin concentrations (data not shown). Increasing valinomycin above 10 μg/mg produced no further increases in chloride flux. For both ghosts and MVV, ~50 μg of membrane protein was used in each measurement of chloride flux. All solutions used in fluorescence experiments were filtered through 0.22-μm Millipore filters (Bedford, MA) to remove dust particles, which scatter light.

The rates of chloride flux in nmol/[s·(mg of membrane protein)] were calculated from the initial rate of change of SPQ fluorescence and the Stern–Volmer quench constant (K). According to the Stern–Volmer relationship

$$F = F_{ex} + F_0/(1 + K[Cl]) \quad (1)$$

where F_{ex} is the time-independent fluorescence of extravesicular SPQ and F_0 and F are the fluorescence intensities of intravesicular SPQ in the presence and absence of chloride. On the basis of Stern–Volmer plots for SPQ quenching by chloride in the presence of a constant gluconate concentration, K (in M^{-1}) was equal to 118/(1 + 0.007[gluconate]), where [gluconate] is in mM. To obtain d[Cl]/dt, eq 1 is differentiated with respect to time

$$(d[Cl]/dt)_{t=0} = F_0[dF/dt]_{t=0}/[K(F - F_{ex})^2] \quad (2)$$

where $[dF/dt]_{t=0}$ is estimated from the initial slope of a single exponential function fitted to the first part of the SPQ fluorescence time course where chloride concentration is known. F_0 and F_{ex} are calculated from eq 1 in which F is evaluated at zero and infinite time:

$$F_0 = (F_i - F_s)(1 + K[Cl]_i)(1 + K[Cl]_s)/[K([Cl]_s - [Cl]_i)] \quad (3)$$

$$F_{ex} = F_i - F_0/(1 + K[Cl]_i) \quad (4)$$

where F_i is F at $t = 0$, F_s is F measured after addition of Triton X-100 to the sample (final concentration 0.01%), and $[Cl]_i$ and $[Cl]_s$ are the known initial and final chloride concentrations. For MVV, flux rates in terms of nmol/(s·mg) were determined by multiplication of d[Cl]/dt (mM/s) by the intravesicular glucose space (3.6 μL/mg of membrane protein; Ruzycki et al., 1978).

In experiments measuring placental vesicle chloride conductance, MVV were loaded with 10 mM SPQ by incubation for 16 h at 4 °C in 250 mM sucrose and 20 mM HEPES–Tris, pH 7.00, containing (a) 100 mM potassium gluconate or (b) 10 mM potassium gluconate, 90 mM NMG–gluconate, and 10 mM SPQ and washed twice in this buffer (minus SPQ) prior to use. MVV (~50 μg of membrane protein) containing 10 μg of valinomycin/mg of membrane protein were added to 250 mM sucrose and 20 mM HEPES–Tris, pH 7.00, containing 50 mM KCl and 50 mM potassium gluconate.

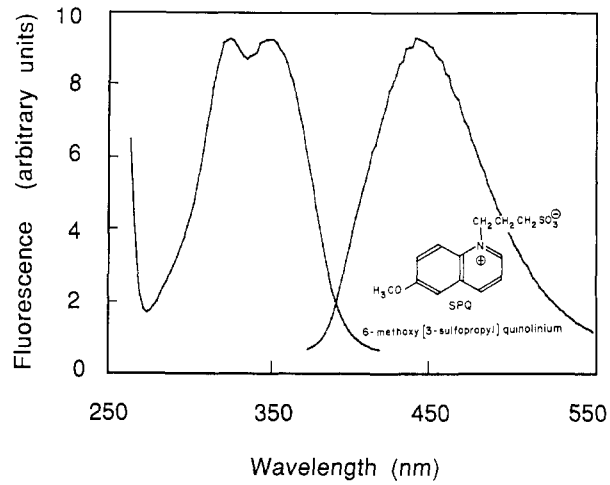

FIGURE 1: Fluorescence excitation and emission spectra of SPQ. Excitation and emission spectra of 10 μM SPQ [6-methoxy-*N*-(3-sulfopropyl)quinolinium] dissolved in water. Also shown is the molecular structure of SPQ.

Chloride influx rates were calculated from the time course of SPQ fluorescence with eq 2–4 and the value for the intravesicular glucose space.

In experiments measuring chloride/sulfate exchange, erythrocyte ghosts (~50 μg of protein) containing 100 mM sucrose, 100 mM KCl, and 50 mM HEPES–Tris, pH 7.00, were mixed with 2 mL of 100 mM sucrose, 66 mM K_2SO_4, and 50 mM HEPES–Tris, pH 7.00, in the presence or absence of 0.1 mM H_2DIDS, and the time course of SPQ fluorescence was followed. Chloride concentrations were calculated from SPQ fluorescence data, transformed with eq 1, 3, and 4. Ghosts used for $^{35}SO_4$ uptake experiments were incubated for 16 h in 100 mM sucrose, 100 mM KCl, and 50 mM HEPES–Tris, pH 7.00, with or without 1 mM SPQ. Ghosts were not washed prior to use. Ghosts (~50 μg of protein) were added to 200 μL of 100 mM sucrose, 66 mM K_2SO_4, and 50 mM HEPES–Tris, pH 7.00, containing tracer quantities of $H_2{}^{35}SO_4$ (0.1 μCi/mL) and incubated at 37 °C for 0–80 s. After various time intervals, uptake was interrupted by the rapid addition of 2 mL of cold stop solution (100 mM NaCl, 50 mM HEPES–Tris, pH 7.00) containing 0.5 mM SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) followed by immediate mixing and filtration on a 3-μm Millipore filter. After being washed three additional times with 2 mL of stop solution, the filter was dissolved in Scinti-Verse Bio HP (Fisher Scientific, Fairland, NJ) for liquid scintillation counting.

RESULTS

The excitation and emission spectra of SPQ are shown in Figure 1 along with the molecular structure of SPQ. Both spectra were broad with maxima at 320 and 350 nm (excitation) and 445 nm (emission). The shapes of the excitation and emission spectra were not altered by the addition of chloride (10–150 mM; data not shown). The quantum yield and extinction coefficient are 0.53 and 4265 M^{-1} cm^{-1}, respectively, as determined by Wolfbeis and Urbano (1982).

The Stern–Volmer plot of SPQ fluorescence quenching by chloride (F_0/F vs. [Cl]) was linear with a quenching constant of 118 M^{-1} (Figure 2). Bromide, iodide, and thiocyanate also showed linear plots at concentrations <15 mM with quenching constants of 197, 282, and 225 M^{-1}, respectively. At concentrations >15 mM, Stern–Volmer plots for bromide, iodide, and thiocyanate showed upward curvature. Analysis of bromide quenching data (0–150 mM) by a combined colli-

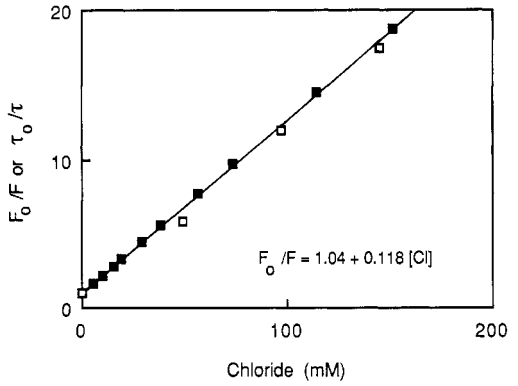

FIGURE 2: Stern–Volmer plot for quenching of SPQ fluorescence by chloride. The fluorescence of 5 μM SPQ in water at 23 °C was measured with increasing [KCl]. F_0/F (filled squares) is plotted against [Cl], where F and F_0 are the fluorescence intensities of SPQ in the presence and absence of Cl. The quenching constant (K = 118 M^{-1}) was calculated from the Stern–Volmer relationship, $F_0/F = 1 + K$[Cl]. The quench constant was also determined at 0.1, 1, and 10 mM SPQ and found not to be significantly different from the value of 118 M^{-1} calculated for 5 μM SPQ. The fluorescence lifetime of SPQ in the presence of Cl was determined by heterogeneity analysis of three frequency phase-modulation lifetime measurements. Lifetime data are plotted as τ_0/τ (open squares) against [Cl], where τ and τ_0 are the SPQ lifetimes in the presence and absence of quencher.

sional and static quenching model (Lakowicz, 1983) gave a static quenching constant of 2.4 M^{-2}. SPQ fluorescence was not quenched by bicarbonate, phosphate, sulfate, sodium, potassium, calcium (0–100 mM), ionic strength (0–400 mM), and pH (5–9). Gluconate, acetate, succinate, and citrate (0–50 mM) quenched SPQ fluorescence slightly with quench constants of 7, 12, 23, and 25 M^{-1}, respectively.

Stopped-flow and lifetime measurements were performed to examine the mechanism of SPQ fluorescence quenching by chloride. In stopped-flow experiments, quenching of SPQ by chloride was complete within the instrumental dead time (2 ms), demonstrating the rapid response of SPQ. In water, ethylene glycol, and ethanol, SPQ exhibited single fluorescence lifetimes of 26.3, 25.7, and 23.9 ns, as determined from the analysis of phase and modulation lifetime data, indicating the presence of a single fluorophore in these solvents. In the presence of chloride, the SPQ lifetime in water remained as a single component, but the lifetime was reduced from 26.3 ns at 0 mM Cl to 1.5 ns at 150 mM Cl (increasing τ_0/τ, Figure 2), demonstrating that changes in fluorescence intensity were due to collisional quenching.

After loading, leakage of SPQ from cells or vesicles was minimal (<5%/h) when maintained at 4 °C and was therefore negligible over the time course of the chloride transport measurements reported here. At 23 °C, the half-time for SPQ leakage from placental microvillous vesicles was ~20 min. No photobleaching of SPQ was apparent with SPQ either in free solution or loaded into cells or vesicles at the incident light levels used experimentally.

Chloride transport was investigated initially in a well-characterized system, the erythrocyte band 3 anion exchanger (Knauf, 1986). Sealed erythrocyte ghosts loaded with 1 mM SPQ and 100 mM Cl were mixed with isoosmotic solutions at 37 °C containing either sulfate or bicarbonate. Both of these compounds are known to exchange for chloride across the erythrocyte membrane with a 1:1 stoichiometry. Addition of ghosts to sulfate caused a rapid increase in fluorescence as intracellular chloride was exchanged for external sulfate and SPQ fluorescence was "unquenched" (Figure 3A). In the presence of H_2DIDS, this increase in fluorescence was dramatically reduced. The chloride efflux rate, calculated from

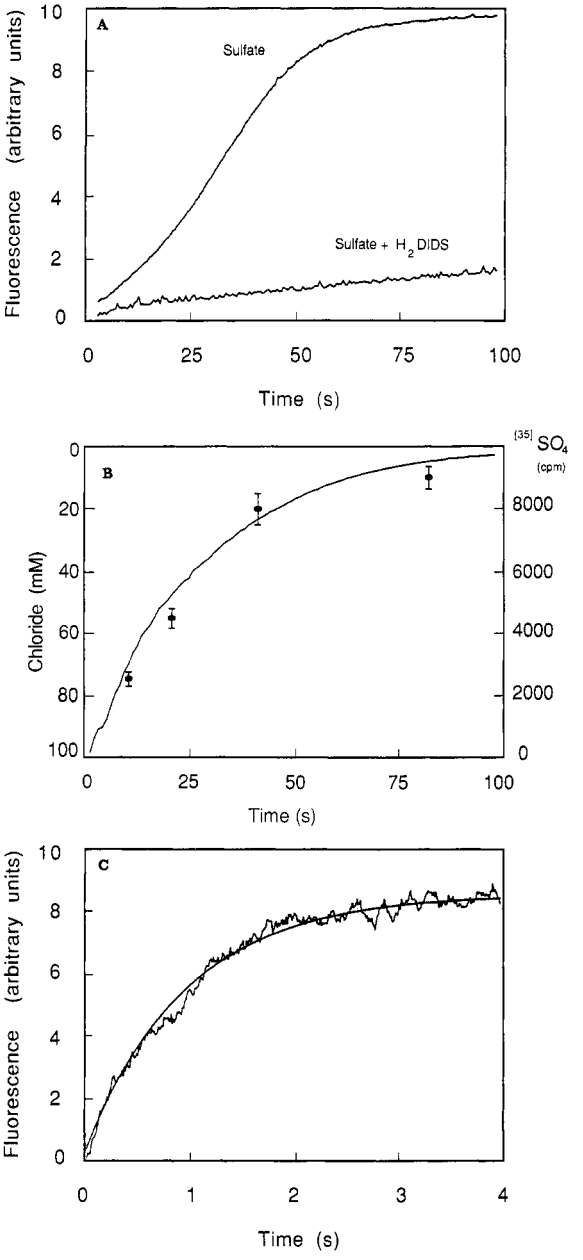

FIGURE 3: Erythrocyte chloride/anion exchange. (A) Representative data showing the changes in SPQ fluorescence intensity when erythrocyte ghosts containing 100 mM Cl and SPQ were added to 66 mM K_2SO_4 or 66 mM K_2SO_4 + 0.1 mM H_2DIDS. (B) Increase in intracellular [Cl] measured using SPQ fluorescence (shown by the continuous line) when ghosts containing 100 mM Cl and SPQ were added to 66 mM K_2SO_4. $^{35}SO_4$ uptake (mean ± SD, n = 3) is plotted at 10, 20, 40, and 80 s (filled symbols). (C) Measurement of the increase in SPQ fluorescence as ghosts containing 100 mM Cl and SPQ were rapidly mixed with 20 mM HCO_3 in the stopped-flow apparatus. The smooth curve is a single exponential fitted to the experimental data with a time constant of 0.87 s.

the changes in intracellular chloride concentration (Figure 3B), was 3.8 ± 0.5 mM/s (mean ± SD, n = 4), which decreased to 0.3 ± 0.2 mM/s in the presence of the stilbene inhibitor, H_2DIDS (0.1 mM). Addition of ghosts containing chloride and SPQ to 25 mM bicarbonate caused a rapid increase in fluorescence, measured with stopped-flow fluorescence (Figure 3C). The chloride efflux rate in the presence of bicarbonate was 21.1 mM/s, which decreased to 0.7 mM/s in the presence of 0.5 mM H_2DIDS.

To test the validity of SPQ measurements and to show that SPQ did not itself alter chloride/sulfate exchange, ghosts plus or minus 1 mM SPQ and containing 100 mM Cl were added

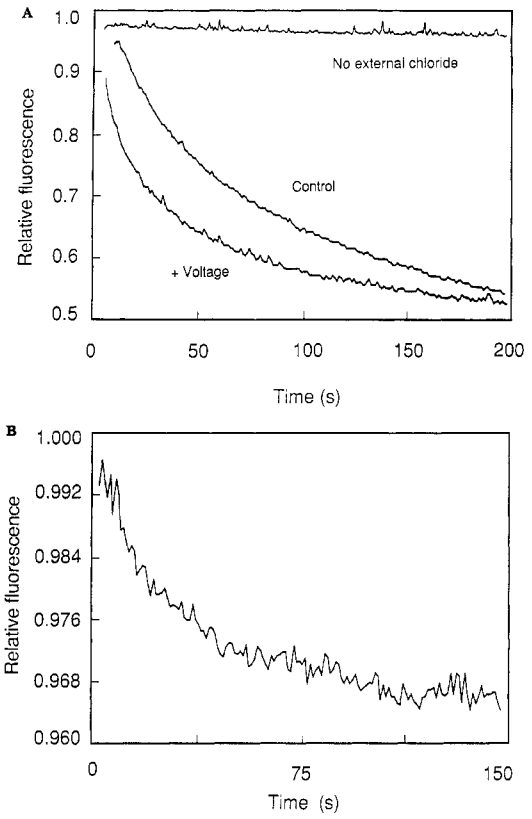

FIGURE 4: Placental vesicle chloride conductance. (A) Representative trace showing the change in SPQ fluorescence as MVV containing SPQ and valinomycin were added to medium containing 50 mM Cl in the absence or presence of a 60-mV membrane potential (positive inside). (B) Time course of SPQ fluorescence when MVV containing SPQ were mixed with medium containing 2 mM Cl.

to sulfate buffer containing tracer $^{35}SO_4$ and incubated at 37 °C in the presence and absence of H_2DIDS. The time course of $^{35}SO_4$ uptake paralleled the appearance of SPQ fluorescence for ghosts in the absence and presence of SPQ (Figure 3B). The initial rate of chloride efflux determined from $^{35}SO_4$ uptake was 3.4 ± 0.4 mM/s (mean ± SD, n = 3) in the absence and 3.5 ± 0.3 mM/s in the presence of SPQ. In the presence of 0.1 mM H_2DIDS, the 80-s uptake of $^{35}SO_4$ was 16% (–SPQ) or 14% (+SPQ) of that observed in the absence of H_2DIDS, similar to results determined by SPQ fluorescence. The similarities in time course, rates, and H_2DIDS inhibition suggest that SPQ fluorescence accurately reflects erythrocyte anion-exchange activity. The similarity between $^{35}SO_4$ uptake in the presence and absence of SPQ demonstrates that SPQ does not affect the exchange process.

To demonstrate application of SPQ fluorescence in a membrane vesicle system, chloride transport was examined in MVV purified from human placenta, which have previously been characterized with respect to water and proton transport and membrane fluidity (Illsley & Verkman, 1986b; Cabrini et al., 1986; Illsley et al., 1987b). MVV containing 100 mM potassium gluconate and loaded with 10 mM SPQ were added to 50 mM KCl and 50 mM potassium gluconate at 23 °C, and the time course of SPQ fluorescence was monitored (Figure 4A). The initial chloride influx rate was 0.61 ± 0.07 nmol/[s·(mg of membrane protein)] (mean ± SD, n = 4). When MVV containing 10 mM potassium gluconate and 90 mM NMG–gluconate were added to the same external solution, the influx rate increased by 65% [1.01 ± 0.11 nmol/(s·mg)], demonstrating that chloride influx is sensitive to K^+/valinomycin-generated membrane potentials. The voltage-driven rate was decreased 40 ± 2% (n = 3) in the presence of 1.0 mM diphenylamine-2-carboxylate, a chloride channel blocker (DiStefano et al., 1985). These data provide strong evidence for a voltage-sensitive chloride conductance in MVV. An identical influx rate was measured when 2 mM SPQ was used in place of 10 mM SPQ to load MVV, indicating that SPQ does not act as a chloride ionophore. In the absence of chloride, SPQ fluorescence did not change with time (<0.1%/s); however in the presence of a small (2 mM) chloride gradient, time-dependent fluorescence quenching of SPQ was measurable (Figure 4B).

Discussion

The studies presented here show that SPQ is a simple yet powerful method to examine chloride transport mechanisms. The response of SPQ fluorescence to chloride is both sensitive and rapid. A point of major importance in biological applications is the fact that, of the physiological anions, SPQ is quenched by only chloride to any significant degree. Organic anions such as succinate, at the concentrations normally present within cells, will produce negligible quenching of SPQ compared to that caused by chloride. The small value of the static quench constant determined for bromide quenching of SPQ suggests that the nonlinear quenching observed at higher bromide, iodide, and thiocyanate concentrations is not caused by a true static mechanism but rather by the "sphere of action" effect (Lakowicz, 1983).

The high water solubility of SPQ permits the use of high fluorophore concentrations in loading cells and vesicles so that high fluorescence signals are possible despite small sample volumes. Loading takes place by transmembrane diffusion, the rate of loading (and conversely of leakage) being proportional to loading temperature. The slow rate of SPQ leakage when cells or vesicles are maintained at 4 °C results in constant intracellular or intravesicular dye concentration over the time course of transport measurements.

The experiments demonstrating stilbene-sensitive chloride/sulfate and chloride/bicarbonate exchange in human erythrocyte ghosts show that SPQ reports the instantaneous cell chloride concentration accurately. These experiments also show that SPQ does not inhibit or activate chloride transport.

The existence of voltage-sensitive chloride transport in human placental microvillous vesicles has been suggested previously in studies using potential-sensitive fluorescence or ^{36}Cl methods (Illsley & Verkman, 1986a; Shennan et al., 1986). The data shown here demonstrate the presence of an inhibitable, voltage-sensitive chloride channel in MVV. In addition, the data show that SPQ does not act as an ionophore or carrier in the membrane. These experiments demonstrate the applicability of the dye to the measurement of electrogenic transport.

In addition to studying chloride transport, the properties of SPQ suggest that this probe may be useful for studies other than those of chloride transport. SPQ may be used for measurement of transport of other halides and possibly organic anions such as citrate and succinate. Of particular interest is the ability to study kinetic aspects of transporters that previously have not been amenable to examination due either to rapid transport rates or to poor time resolution of existing methods (e.g., erythrocyte chloride/bicarbonate exchange at 37 °C; Figure 3B). In renal cortical brush border vesicles from rabbit, we have used SPQ to demonstrate a chloride conductance, whereas in basolateral membranes we have shown the presence of stilbene-inhibitable chloride/anion exchange and coupled sodium chloride transport (Illsley et al., 1987a).

The applications of chloride transport measurement by SPQ are not limited to vesicle systems but are likely to include

measurements of chloride concentration and transients in cultured cells and in intact epithelia. One area in which this method may prove immediately useful is in cystic fibrosis research, where the suspected pathological lesion is a defect in the chloride conductance of tracheal epithelial cells (Welsh, 1986; Frizzell et al., 1986). A rapid and reliable assay for the chloride conductance that requires only a small quantity of tissue may be possible with SPQ.

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Articles

Identification of Subunits of Acetylcholine Receptor That Interact with a Cholesterol Photoaffinity Probe†

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ABSTRACT: All four subunits of the acetylcholine receptor in membrane vesicles isolated from *Torpedo californica* have been labeled with [^{3}H]cholesteryl diazoacetate. As this probe incorporates into lipid bilayers analogously to cholesterol, this result indicates that acetylcholine receptor interacts with cholesterol. This investigation also demonstrates that this probe is a useful reagent for studying the interaction of cholesterol with membrane proteins.

The nicotinic acetylcholine receptor (AChR)[1] is an integral membrane protein that transduces a signal mediated by ACh into a membrane depolarization. This depolarization is the result of a transient current caused by the opening of an ion channel [for recent review, see Adams (1981)]. A unique opportunity to correlate the molecular structure of a gated ion channel with its functional properties is afforded by the availability of membrane vesicles greatly enriched in AChR from *Torpedo* electric organ [for recent reviews, see Conti-Tronconi and Raftery (1982) and Raftery et al. (1983)].

The AChR isolated from *Torpedo californica* is a pentameric complex of homologous subunits with apparent M_r of 40K, 50K, 60K, and 65K in a stoichiometry of 2:1:1:1 (Raftery et al., 1980). Several experimental approaches have shown that *T. californica* AChR is a transmembrane protein. Extracellular exposure of AChR was demonstrated by using anti-AChR antibodies conjugated with ferritin visualized by electron microscopy (Karlin et al., 1978). In other electron microscopic studies, antigenic determinants were revealed on both sides of the electroplax membrane (Strader et al., 1979; Tarrab-Hazdai et al., 1978). Exposure of the 40K, 50K, and 60K subunits to the aqueous phase was shown by labeling with

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[1] Abbreviations: AChR, acetylcholine receptor; ACh, acetylcholine; BrACh, bromoacetylcholine; MBTA, (4-*N*-maleimidobenzyl)trimethylammonium iodide; ESR, electron spin resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; α-BgTx, α-bungarotoxin; THF, tetrahydrofuran; TEA, triethylamine; UV, ultraviolet; TLC, thin-layer chromatography; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; IR, infrared.

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