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Measurement of intracellular chloride ion concentration in ICC *in situ* and in explant culture

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Abstract

Background *Chloride channels are proposed to play a central role in the electrical pacemaking mechanism of interstitial cells of Cajal (ICC). A key unknown factor in the consideration of this role is the chloride equilibrium potential (E_{Cl}), as determined by the relative concentrations of intra- ($[Cl^-]_i$) and extracellular ($[Cl^-]_o$) chloride ions.* ***Methods*** *To calculate the E_{Cl} of ICC, $[Cl^-]_i$ was measured with the fluorescent chloride indicator N-(6-methoxyquinolyl) acetoethyl ester (MQAE). Pacemaker ICC in explant cultures or in situ, i.e. ICC associated with the myenteric plexus of the small intestine, were loaded with MQAE and fluorescence was measured by laser scanning confocal microscopy. The dye fluorescence was then calibrated against known $[Cl^-]_i$ by treating the explants or in situ preparations with chloride ionophore and varying bath chloride concentrations.* ***Key Results*** *In explants, ICC $[Cl^-]_i$ was measured as 13 mmol L^{-1} with $[Cl^-]_o$ of 100 mmol L^{-1}, giving an E_{Cl} of −23 mV. With $[Cl^-]_o$ at 166 mmol L^{-1}, $[Cl^-]_i$ was 26 mmol L^{-1}, giving an E_{Cl} of −21 mV. In situ, ICC $[Cl^-]_i$ was measured as 26 mmol L^{-1} with $[Cl^-]_o$ of 130 mmol L^{-1}, giving an E_{Cl} of −18 mV. Importantly ICC compensate for changes in extracellular chloride by changing $[Cl^-]_i$ and thus maintain E_{Cl}. In ICC explant clusters, $[Cl^-]_i$ was seen to fluctuate, possibly evoked by rhythmic changes in intracellular calcium.* ***Conclusions & Inferences*** *The intracellular chloride concentration in ICC fluctuates to keep its equilibrium potential constant. The identification of E_{Cl} as positive to the resting membrane potential of ICC indicates that opening of chloride channels will depolarize ICC.*

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INTRODUCTION

Chloride channels have long been considered as just 'background' channels but recently they have been implicated in important physiological and pathophysiological processes related to blood pressure regulation, muscle tone, volume regulation, synaptic transmission, and cellular excitability.[1] The determination of physiological roles for chloride channels is hampered by our limited knowledge at the molecular level, by non-specificity of pharmacological agents affecting the channels and by the fact that the chloride equilibrium potential (E_{Cl}) is often not known because the exact intracellular chloride concentration is not known. Depending on whether E_{Cl} is higher or lower than the resting membrane potential, opening of chloride channels will depolarize or hyperpolarize the cell, respectively. This can be critical to physiological function. For instance in many neurones E_{Cl} is just positive to the resting membrane potential in the embryo, but postnatally shifts to potentials negative of the resting membrane potential, with the consequence that activation of the $GABA_A$ receptor (a chloride channel) is initially excitatory (depolarizes the cell, encouraging action potentials) then inhibitory.[2]

Several studies have implicated chloride channels in the generation of inward (depolarizing) pacemaker current by ICC.[3–16] Because of the uncertainties mentioned affecting physiological and electrophysiological studies, the exact role of chloride channels in shaping membrane potentials may remain somewhat speculative. We set out to remove one important variable, the E_{Cl} of ICC. For chloride channels to contribute to inward pacemaker current in ICC, E_{Cl} must be positive to the resting membrane potential (typically in the range of −70 to −50 mV). We calculated the E_{Cl} of ICC in explant culture (a preparation used for patch clamp studies) and *in situ* preparations (in association with the myenteric

plexus), by measuring $[Cl^-]_i$ with a chloride-quenchable fluorescent dye, *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE).[17] In addition, we were interested to know whether or not E_{Cl} was affected by changes in $[Cl^-]_o$, as this is often a variable in the study of chloride channels.

MATERIALS AND METHODS

Tissue preparation and cell culture

All procedures were carried out in accordance with regulations from the Animal Ethics Board of McMaster University. For *in situ* preparations the jejunum was dissected from female CD1 mice between 5 and 6 weeks of age and a longitudinal myenteric muscular preparation (LMMP) was made as described elsewhere.[14] Explant cultures were made as described elsewhere[6] and were used after 6 days of culture.

MQAE measurements

All experiments were carried out in either Normal Tyrode solution [NT, mmol L^{-1}: 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 2 $Ca(NO_3)_2$, 1 $MgSO_4$, 5 HEPES, 5.5 glucose; to pH 7.35 with HCl], Isethionate Tyrode solution (IT, as for NT but with Na-isethionate replacing NaCl) or a mixture of the two, at room temperature. With the *in situ* preparation, solutions were bubbled with 95% O_2 / 5% CO_2. Some experiments were carried out using SCN as a substitute for Cl, the results were similar to replacement of Cl with Iso and are not included in the data presented.

In situ preparations or explant cultures were loaded with 5 mmol L^{-1} MQAE, in IT, for 1 h at room temperature. Thereafter, it was rinsed with chloride-free solution for 1 h and 40 min. N-(6-methoxyquinolyl) acetoethyl ester fluorescence was imaged with a Zeiss laser scanning confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada). In each image regions of interest (ROIs) were selected in ImageJ (NIH, Bethesda, MD, USA), corresponding to ICC identified by morphology or ACK4 co-staining (see below). With each tissue, six independent areas were chosen and in each area, eight well positioned ICC were selected for analysis. The mean fluorescence value from all tissues or all cell cultures subjected to specific conditions was then used as the basis for further analysis (see below). Once MQAE fluorescence had been measured under control conditions (with physiological bath chloride concentration), MQAE fluorescence was calibrated against $[Cl^-]_i$ by treating the sample with 0.1 mmol L^{-1} ionophore I (Sigma Aldrich Canada Ltd., Oakville, ON, Canada) and varying bath $[Cl^-]$ by adjusting the ratio of NT to IT. Calibration data were fitted with the Stern–Volmer equation.[17]

$$F_0/F([Cl^-]_i) = 1 + [Cl^-]_i.K_{sv}$$

where F is the mean ROI fluorescence value (arbitrary units); $[Cl^-]_i$ is the bath chloride concentration (mmol L^{-1}); $F_0 = F([Cl^-]_i = 0)$ and K_{sv} is the Stern–Volmer constant ($mmol^{-1}$ L). Fitting was performed with Origin software (OriginLab, Northampton, MA, USA).

C-kit staining

In some experiments the sample was co-loaded with Cy3-conjugated ACK4 (1 : 50), a monoclonal antibody against the c-kit receptor, for 45 min at room temperature before washing with NT.

Materials

ACK4 monoclonal antibody was purchased from Cedarlane (Burlington, ON, Canada). *N*-(6-methoxyquinolyl) acetoethyl ester was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma (Oakville, ON, USA).

RESULTS

ICC *in situ*

To identify ICC *in situ*, the circular muscle was removed from the external muscle layers of the jejunum to expose the myenteric plexus with associated pacemaker ICC (LMMP; see Methods). Staining using anti-c-kit (ACK4) antibody identified the ICC. Regions of Interest used to measure MQAE fluorescence were c-kit positive cell bodies.

The results of the calibration of MQAE fluorescence against $[Cl^-]_i$ are shown in Fig. 1. As the bath chloride concentration (equal to $[Cl^-]_i$ in the presence of ionophore) was increased by replacing NaIso with NaCl, MQAE fluorescence decreased (Fig. 1A). The quenching of MQAE by chloride can be described by the Stern–Volmer equation.[17] When the MQAE fluorescence of ICC were plotted against $[Cl^-]_i$ according to this relationship, a clear linear relationship was apparent (Fig. 1B). When fitted with the Stern–Volmer equation this gave Stern–Volmer constant (K_{SV}) of 0.04 $mmol^{-1}$ L (Table 1).

Given the K_{SV} for a preparation, the $[Cl^-]_i$ prior to ionophore calibration (i.e. under physiological conditions) can be calculated from MQAE fluorescence according to the Stern–Volmer equation. Furthermore, given this calculated value of $[Cl^-]_i$ and the experimental value of $[Cl^-]_o$, E_{Cl} can be calculated according to the Nernst equation,

$$E_{Cl} = (RT/F).\ln([Cl^-]_i/[Cl^-]_o)$$

Calculated values of E_{Cl} were −22.7 mV with $[Cl^-]_o$ = 100 mmol L^{-1} and −20.5 mV with $[Cl^-]_o$ = 166 mmol L^{-1} (Table 1).

Explant ICC

Isolated ICC were identified in explant cultures at the edges of explants. The cell bodies of ICC were chosen as regions of interest for MQAE fluorescence measurements. The results of the calibration of MQAE

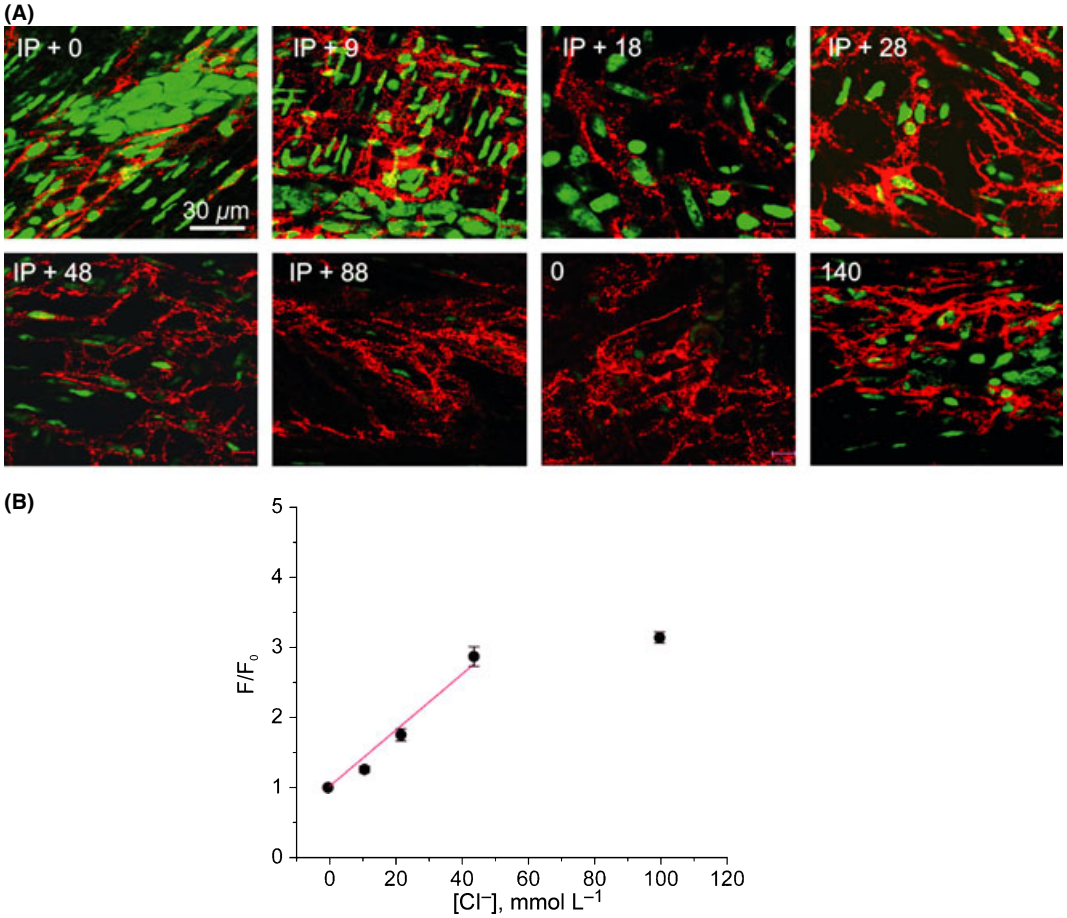

Figure 1 Calibration of *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE) fluorescence in *in situ* preparations. (A): raw confocal images of MQAE fluorescence (green) and ACK4 staining (red) with various bath [Cl^-] (indicated by number in top left of each frame; IP indicates presence of ionophore). (B): Stern–Volmer plot of calibration data. Each plotted value represents the mean of 8 –16 regions of interest from the same preparation. Standard errors are indicated by vertical bars. The red line is the fit of the Stern–Volmer equation to the individual data points (not the mean values), with K_{SV} = 0.0402 ± 0.0018 and R^2 (correlation coefficient) = 0.80. Fit did not include last data point ($[Cl^-]_i$ = 100 mmol L^{-1}) because at this value MQAE fluorescence was below background.

Table 1 Summary of calculations of MQAE data

Preparation	$[Cl^-]_o$, mmol L^{-1}	F_0/F	K_{sv}, mmol^{-1} L	$[Cl^-]_i$, mmol L^{-1}	E_{Cl}, mV
In situ	100	1.515	0.04019	12.80	–22.70
	166	2.039	0.04019	25.85	–20.54
Explant	130	2.683	0.06451	26.09	–17.74

MQAE, *N*-(6-methoxyquinolyl) acetoethyl ester; $[Cl^-]_o$ bath chloride concentration under physiological conditions (without ionophore); *F*, corresponding MQAE fluorescence; F_0, MQAE fluorescence with ionophore and zero bath chloride; K_{sv}, Stern–Volmer constant; $[Cl^-]_i$, intracellular chloride concentration calculated from the Stern–Volmer equation ($[Cl^-]_i = K_{sv}.(F_0/F - 1)$); E_{Cl} chloride equilibrium potential calculated according to the Nernst equation (T = 295.15 K, R = 8.314 J K^{-1} mol^{-1}, F = 96488 C mol^{-1}).

fluorescence against $[Cl^-]_i$ are shown in Fig. 2. As the bath chloride concentration (equal to $[Cl^-]_i$ in the presence of ionophore) was increased by replacing NaIso with NaCl, MQAE fluorescence decreased (Fig. 2A). When the MQAE fluorescence of ICC were plotted against $[Cl^-]_i$ according to the Stern–Volmer equation, a linear relationship was observed (Fig. 2B) giving a Stern–Volmer constant (K_{SV}) of 0.065 $mmol^{-1}$ L (Table 1). This gave a calculated E_{Cl} of –17.7 mV with $[Cl^-]_o$ = 130 mmol L^{-1} under physiological conditions, prior to ionophore calibration (Table 1).

With some explant cultures, prominent networks of ICC develop at the edge of the explant. Such networks ICC have a high chance of showing robust rhythmic pacemaker activity. In such networks MQAE fluorescence was seen to spontaneously fluctuate, demonstrating rhythmic decreases in $[Cl^-]_i$ (Fig. 3). In three cultures the rhythmic activity was regular at a frequency of 11 ± 2 min^{-1}.

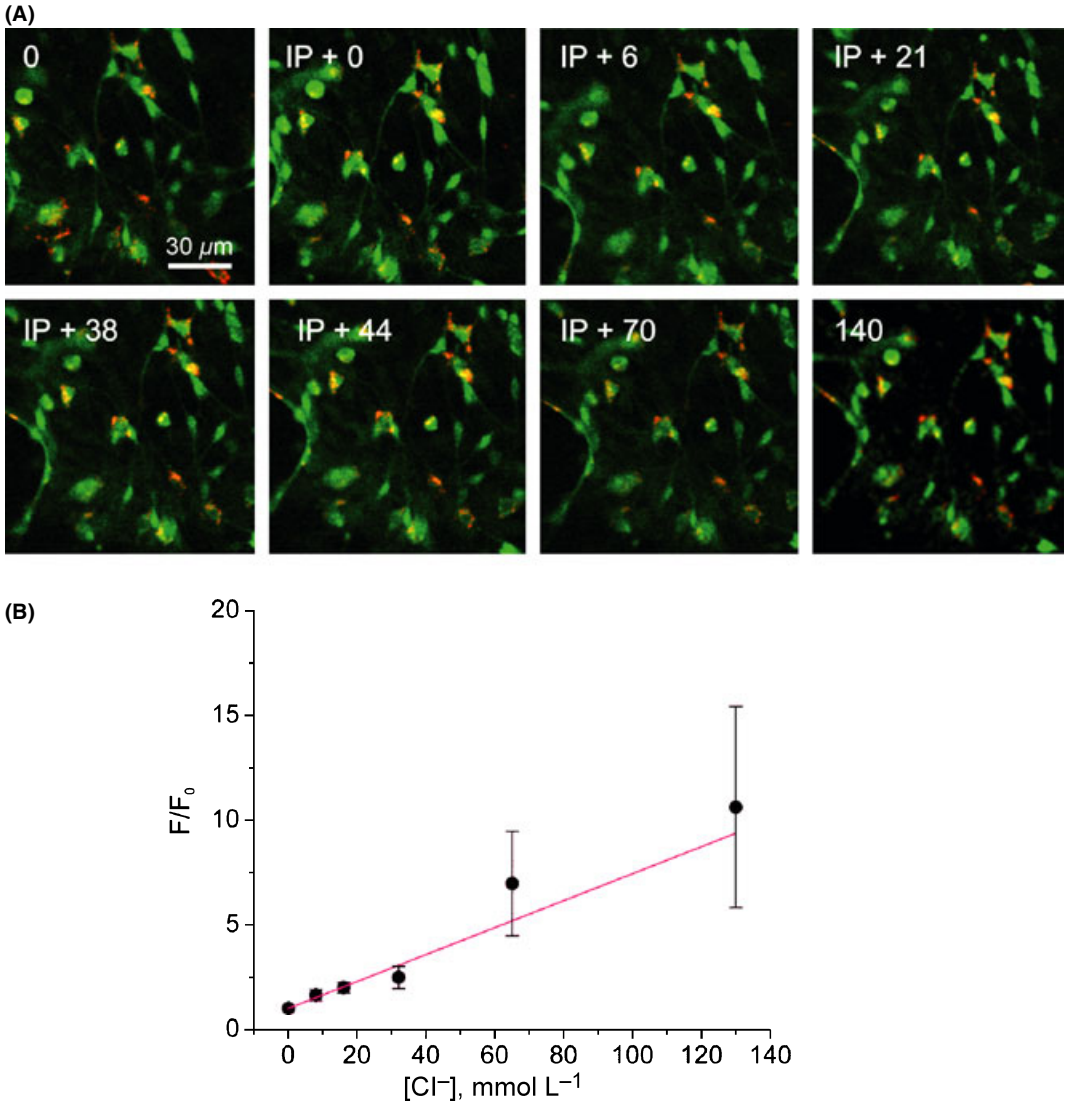

Figure 2 Calibration of *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE) fluorescence in explant cultures. (A): raw confocal images of MQAE fluorescence (green) and ACK4 staining (red) with various bath $[Cl^-]$ (indicated by number in top left of each frame; IP indicates presence of ionophore). (B): Stern–Volmer plot of calibration data. Each plotted value represents the mean of two to four regions of interest from different explants (assessed from six independent areas of each tissue). Standard errors are indicated by vertical bars. The red line is the fit of the Stern–Volmer equation to the individual data points (not the mean values), with K_{SV} = 0.0645 ± 0.0088 and R^2 (correlation coefficient) = 0.61.

DISCUSSION

The present study shows that MQAE can reliably be used to measure $[Cl^-]_i$ in ICC and thereby calculate E_{Cl}. The values of E_{Cl} for ICC *in situ* were consistent over a wide range of $[Cl^-]_o$, indicating that the cell compensates for changes in extracellular chloride by changing $[Cl^-]_i$ and thus maintaining E_{Cl}. This homeostasis of E_{Cl} suggests a physiological importance for chloride currents beyond simple charge equality (i.e. as a counter ion against cations).

The E_{Cl} for explant ICC was only slightly higher than that for ICC *in situ* – 17.7 mV *vs* – 21 mV, respectively. These values are comparable with values measured in smooth muscle by ion-selective microelectrodes [see table 3 of Frings *et al.* (18)]. They also imply that any chloride currents in ICC are depolarizing, as suggested by several current-clamp microelectrode studies of phenomena associated with ICC pacemaking – slow waves, driving potentials and unitary potentials.[5,8] The explant data indicate that , such currents can actually be detected by MQAE fluorescence. This was shown to transiently rise, corresponding to transient decreases in $[Cl^-]_i$. If this decrease in $[Cl^-]_i$ is caused by extrusion to the extracellular space, this corresponds to an inward chloride

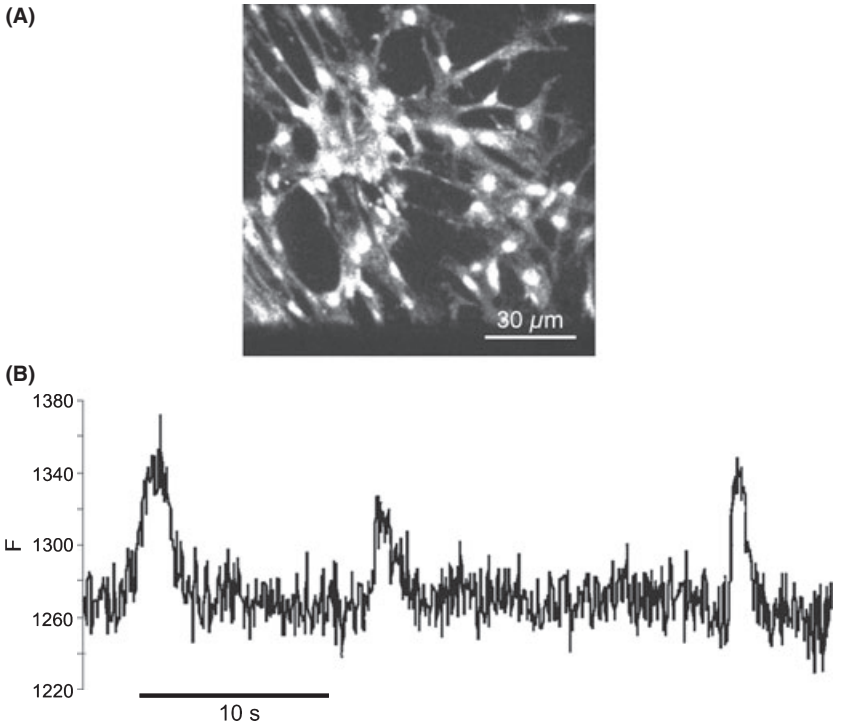

Figure 3 (A) Spontaneous transients in *N*-(6-methoxyquinolyl) acetoethyl ester fluorescence in an explant interstitial cells of Cajal. (B) Plotted values represent the mean of regions of interest.

current which would be expected with E_{Cl} positive to the resting membrane potential (as it indeed is). Thus, MQAE could present a method of recording chloride currents in situations where the usual methods (i.e. patch-clamp) are inappropriate or difficult to apply.

We observed that the chloride concentration can rhythmically fluctuate. As it has been established that intracellular calcium fluctuates in ICC related to pacemaking activity[19] it is tempting to suggest that chloride channels may respond to intracellular calcium fluctuations. Calcium-activated chloride channels, those that may respond to fluctuating calcium, include the ANO1 channel[3,7,15] and the high conductance chloride channel.[6,10,14] It will be interesting in the future to assess whether chloride fluctuations are limited to the cytoplasm or whether they also occur within the sarcoplasmic reticulum or mitochondria. The current experiments were executed at 63 × magnification that does not allow reliable differentiation between the cellular structures.

Fluorescence quenching can occur by a wide variety of mechanisms, which have been broadly split into two categories. Static quenching occurs when the fluorescent molecule (fluorophore) and quencher form a stable, non-fluorescent complex. It is often seen when high concentrations of fluorophore lead to foation of fluorophore – fluorophore complexes, and thereby auto-quenching. Dynamic (or 'collisional') quenching occurs by energy transfer during collision of freely diffusible fluorophore and quencher. This covers a whole range of quantum mechanical mechanisms, most of which are still a matter of active research for any particular fluorophore–quencher pair. For aromatic fluorophores (including quinoliniums such as MQAE) and halides this involves charge (electron) transfer from the quenching anion to the aromatic.[20]

Whether the quenching mechanism is static or dynamic, if it involves a single fluorophore and single quencher in a single reaction, it can be described by the Stern–Volmer equation.[21] For static quenching

$$K_{sv} = ([F]^{T} - [F])/([F][Q])$$

where $[F]^{T}$ is the total concentration of fluorophore (free and bound to quencher), $[F]$ is the concentration of unbound fluorophore, and $[Q]$ is the concentration of quencher. For dynamic quenching,

$$K_{sv} = k_{q}\tau_{0}$$

where k_q is the quenching constant and t_0 the excited state lifetime of the fluorophore. Static and dynamic quenching are typically distinguished by the dependence of K_{sv} on temperature, solution viscosity, and other variables.

We have shown in this study that the intracellular chloride concentration changes when marked changes in extracellular chloride occur. This means that in experimental conditions where extracellular chloride is changed to affect the concentration gradient of

chloride, it cannot be assumed that the gradient changes in strict relation to changes in extracellular chloride.

Intracellular chloride concentrations are likely affected by pumps, exchangers, and ion channels. The present study indicates that the intracellular chloride concentration might not be constant under physiological conditions in contrast to what is usually assumed. This means that the chloride concentration gradient across the cell membrane can fluctuate influencing the kinetics of current flow through ion channels.

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