Biochimica et Biophysica Acta, 1153 (1993) 262–266

BBAMEM 76154

Determination of the intracellular free chloride concentration in rat brain synaptoneurosomes using a chloride-sensitive fluorescent indicator

A. Christine Engblom * and Karl E.O. Åkerman

Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, P.O. Box 66, SF-20521, Åbo (Finland)

(Received 5 April 1993)
(Revised manuscript received 24 August 1993)

Key words: Chloride; Chloride-sensitive indicator; Barbiturate; Synaptoneurosome

The chloride-sensitive fluorescent indicator MQAE (*N*-(6-methoxyquinolyl) acetoacetyl ester) has been used for determination of the intracellular free chloride concentration in rat brain synaptoneurosomes. Loading of the synaptoneurosomes with MQAE occurs by transmembrane diffusion. Calibration of the intracellular MQAE was done by determining the correlation between fluorescence intensity and intrasynaptoneurosomal Cl^- concentration in the presence of the Cl^-/OH^- exchanger tributyltin and the K^+/H^+ exchanger nigericin, starting from zero Cl^- concentration. The total quenchable signal of MQAE was determined by adding KSCN in the presence of the K^+ ionophore valinomycin. The correlation between the reciprocal of the fluorescence intensity and the chloride concentration was linear at least up to 50 mM Cl^-. The fluorescence of freshly prepared synaptoneurosomes was then measured and the obtained value was plotted into the calibration curve and the corresponding Cl^- was read. The mean intrasynaptoneurosomal chloride concentration was 14 ± 4 mM. We also quantitatively estimated the Cl^- flux after addition of the barbiturate, pentobarbitone that opens $GABA_A$ receptor-Cl^--channels, to the synaptoneurosomes. An addition of 1 mM pentobarbitone corresponded to an approx. 0.59 mM change in the intrasynaptoneurosomal free chloride concentration. The results show that the chloride-sensitive fluorescent indicator MQAE is a useful tool when determining intracellular chloride activity, and in quantitative determination of chloride fluxes in living cells and subcellular preparations.

Introduction

There is accumulating evidence that Cl^- is not passively distributed over the cell membrane, but is regulated by transport mechanisms [1,2]. The Cl^-/HCO_3^- exchange system is responsible for intracellular pH regulation [3] and cell volume is dependent upon the $K^+/Na^+/Cl^-$ cotransport system and other transport systems (For reviews see Refs. 4,5). The chloride homeostasis is also regulated by Ca^{2+}-dependent Cl^- channels [6], voltage-gated Cl^- channels [7–9], and ATP-driven Cl^- pumps [10]. The intracellular chloride concentration is particularly important in excitable cells, since the Cl^- permeability through $GABA_A$ and glycine receptor Cl^- channels as well as other Cl^- channels regulates cellular excitability. The intracellular chloride activity is needed to determine the Cl^- equilibrium potential. $GABA_A$ receptor-linked Cl^- fluxes have previously been measured using $^{36}Cl^-$ in synaptoneurosomes [11–14], which is a pre- and postsynaptic metabolically intact membrane preparation [11]. The use of fluorescent probes is gaining wider applicability in the study of membrane transport. We have earlier investigated $GABA_A$-, barbiturate-, and ethanol-induced Cl^- fluxes in rat cerebellar granule cells [15–17] and the effect of ethanol on $GABA_A$ and glycine receptor-coupled Cl^- fluxes in rat brain synaptoneurosomes [18], using a chloride-sensitive fluorescent indicator SPQ [19]. The fluorescence of SPQ is quenched by Cl^- by collisional quenching. SPQ has also been used for estimation of intracellular chloride activity in isolated rabbit proximal tubules [20]. One of the main problems using fluorescent probes is calibration and quantitation of signals. Therefore a comparison of obtained changes in many instances and with different preparations, e.g., from different animal strains, is impossible. The aim of this study was to design a calibration protocol for the determination of $GABA_A$ receptor coupled changes in rat brain synaptoneurosomes using a Cl^--sensitive fluorescent indicator MQAE [21] with higher Cl^- sensitivity and fluorescence yields as compared to SPQ.

* Corresponding author. Fax: +358 21 654745.

Experimental procedures

Preparation of synaptoneurosomes. Synaptoneurosomes comprise a subcellular membrane preparation with resealed, intact pre- and postsynaptic parts. The preparation retains receptor-mediated properties and maintain an electrochemical gradient [11,22,23].

The preparation of synaptoneurosomes was performed as described by Schwartz et al. [11], with some modifications [18,23]. Briefly, a young Wistar rat was decapitated and the brain placed in an ice-cold, balanced salt solution (BSS) (composition in mM: NaCl, 137; KCl, 5; $NaHCO_3$, 4.2; $MgCl_2$, 1.0; KH_2PO_4, 0.44; 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonate (TES), 20 (pH 7.4)) to which 1 mM Mg-ATP and 0.1% bovine serum albumin (BSA) had been added. The brain was cut into small pieces (2–3 mm) and manually homogenised (6 strokes) with a loosely fitting, glass-Teflon homogenizer. The homogenate was passed through a nylon mesh (80 μm), and the filtrate was subsequently passed through a cellulose nitrate filter (8 μm) followed by centrifugation at $1000 \times g$ for 15 min. The pellet was washed once in BSS and centrifuged. All the procedures were performed at 4°C.

Electron microscopy examination of synaptoneurosomes prepared in this way, shows that the preparation contains cross-sections of nerve terminals with synaptic vesicles. Many axon terminals makes contact with a postsynaptic structure. Density gradient centrifugation shows that the preparation is virtual absent of other particles and contains very little myelin [23].

Loading synaptoneurosomes with MQAE. The pellet was resuspended in 5 mM MQAE (Lambda Probes & Diagnostics, Austria) dissolved in the balanced salt solution with 10 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA and loaded for 30–45 min at 37°C. After loading, the suspension was centrifuged at $1000 \times g$ for 15 min at 4°C. The pellet was resuspended in BSS with glucose and BSA, and the suspension was kept on ice. An appropriate amount was subsequently centrifuged for each experiment.

Calibration of intrasynaptoneurosomal MQAE. The pellets were resuspended in an experimental medium free of Cl^- (NaCl was substituted by equimolar amounts of D-glucuronic acid, $MgCl_2$ by $MgSO_4$, KCl by $KHSO_4$ (pH 7.4) with NaOH), centrifuged once and resuspended in a cuvette with 350 μl of the Cl^- free medium plus 10 mM glucose and 0.1% BSA. The fluorescence was monitored using a Hitachi F-4000 fluorescence spectrophotometer (excitation 355 nm, emission 460 nm) in a stirred and thermostated cuvette (37°C). Stirring was used to ascertain the rapid and even distribution of the added components.

Calibration of intracellular MQAE was performed by determining the correlation between fluorescence intensity and Cl^- concentration by a procedure modified from those described by Krapf et al. [20] for perfused tissues and Eidelman and Cabantchik [24] for vesicles. The maximal fluorescence (F_{max}) in the absence of Cl^- was obtained by depleting intrasynaptoneurosomal Cl^- by adding the ionophores nigericin (7 μM) and tributyltin acetate (TBT) (10 μM) to the synaptoneurosomes in the chloride free experimental medium. Nigericin is a K^+/H^+ antiporter and removes the H^+ and OH^- gradients. Tributyltin, being an OH^-/Cl^- antiporter [25], equalizes the Cl^- gradient. Four different concentrations of NaCl (between 5 and 50 mM) were then added to every pellet and the fluorescence intensities were noted. At the end of each experiment 150 mM KSCN, in the presence of 5 μM of the K^+ ionophore valinomycin, was added to quench the fluorescence of MQAE. The SCN^- ion has a much higher affinity for the indicator than the Cl^- ion and therefore quenches most of the MQAE fluorescence.

The total quenchable signal (F_t) was obtained by subtracting the fluorescence after addition of KSCN from F_{max} ($F_t = F_{max} - F_{KSCN}$). The change in fluorescence after addition of a given concentration of Cl^- was defined as F_t/F_{Cl} where F_{Cl} is the fluorescence at the given Cl^- concentration minus F_{KSCN}.

Estimation of the intrasynaptoneurosomal free chloride concentration. Freshly prepared synaptoneurosomes were resuspended in the chloride free experimental medium and the fluorescence intensity was rapidly measured. 7 μM nigericin and 10 μM TBT were added to obtain F_{max} and 150 mM KSCN and 5 μM valinomycin were added to obtain F_t. The F_t/F_{Cl} values of the synaptoneurosomes were calculated as described above.

Quantitative determination of chloride fluxes. Synaptoneurosomes were resuspended in the chloride free experimental medium, with 1 mM furosemide added to prevent Cl^- fluxes through the $Na^+/K^+/2Cl^-$ cotransporter and the HCO_3^-/Cl^- exchange system [3,18]. The suspension was centrifuged and the pellet was resuspended in a cuvette containing chloride free experimental medium with 10 mM glucose, 1 mM $CaCl_2$, 0.1% BSA and 1 mM furosemide. Different concentrations (0.01–1.00 mM) of the barbiturate pentobarbitone were added and the fluorescence intensities were recorded. F_{max} and F_t were determined at the end of every experiment as described above by adding nigericin and TBT, and valinomycin and KSCN, respectively, and the F_t/F_{Cl} values corresponding to the different concentrations of pentobarbitone were calculated. The remaining basal leakage of chloride from the synaptoneurosomes in the presence of furosemide [18] was excluded from the calculations. The medium low in Cl^- concentration was used as experimental medium to be able to study outward-directed Cl^- movements [16].

Results

Intrasynaptoneurosomal MQAE calibration and estimation of chloride concentration

In order to be able to quantitate fluorescence changes, an estimate of the total quenchable signal (F_t) has to be obtained. Fig. 1 shows the increase in MQAE fluorescence upon addition of nigericin and tributyltin to synaptoneurosomes in a Cl^- free medium, followed by quenching of the signal by KSCN.

The fluorescence of MQAE was calibrated as described in Experimental procedures by adding chloride to the final concentrations of 0, 5, 10, 20, 30, 40, and 50 mM in the presence of nigericin and tributyltin. A typical experiment is shown in Fig. 2. The data were plotted as a correlation curve (Fig. 3). The relationship between the reciprocal of the intracellular MQAE fluorescence intensity and Cl^- concentration was linear at least up to 50 mM Cl^- ($r = 0.99983$). The fluorescence signal of an indicator affected by collision quenching with substrate, in this case chloride, follows the Stern–Volmer equation:

$$F_t / F_{Cl} = 1 + [Cl^-]/K_{SV}$$

where F_t is the total quenchable fluorescence and F_{Cl} is the proportional fluorescence at a certain Cl^- concentration. The intrasynaptoneurosomal constant K_{SV} for quenching of MQAE by chloride was calculated according to the above equation to be 15.7 mM.

The mean F_t/F_{Cl} value, obtained as shown in Fig. 1 for five different preparations of synaptoneurosomes (3–6 experiments per preparation) varied between 1.61 and 2.28, the mean value was 1.89 ±0.25 (S.E.) (Table I). When the data were plotted into Fig. 3 or fitted into the Stern–Volmer equation with $K_{SV} = 15.7$ mM, the corresponding intrasynaptoneurosomal Cl^- concentration varied between 9.6 mM and 20.1 mM, the mean value was 14.0 ± 3.9 mM (S.E.) Cl^- (Table I).

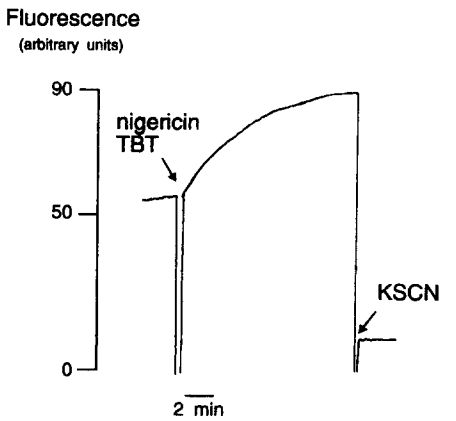

Fig. 1. Estimation of the total quenchable fluorescence (F_t). The fluorescence of the synaptoneurosomes was measured. Maximal fluorescence (F_{max}) was obtained by adding nigericin (7 μM) and tributyltin (10 μM). At the end of the procedure intrasynaptoneurosomal MQAE was quenched by KSCN (150 mM) in the presence of valinomycin (5 μM) to determine the total quenchable signal (F_t).

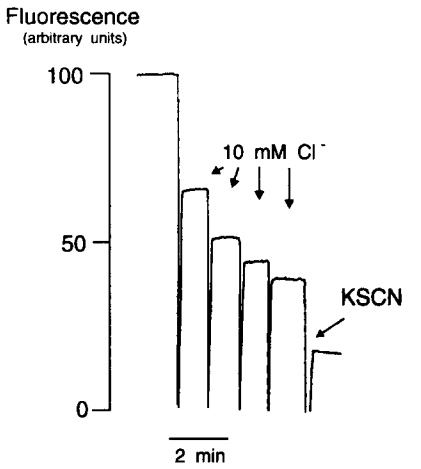

Fig. 2. Intracellular MQAE fluorescence upon additions of Cl^- to rat brain synaptoneurosomes. Cl^- was added in every experiment to synaptoneurosomes in a chloride free medium, in the presence of nigericin and tributyltin (see methods). At the end of each experiment, the total quenchable signal (F_t) was determined by adding KSCN (150 mM) in the presence of valinomycin (5 μM).

Quantitative determination of pentobarbitone-induced chloride fluxes

Addition of pentobarbitone increases the fluorescence of intrasynaptoneurosomal MQAE due to efflux of Cl^- (Fig. 4). The F_t value was obtained by subsequent additions of nigericin, TBT, and KSCN as in Fig. 1. The obtained F_t/F_{Cl} value for different concentra-

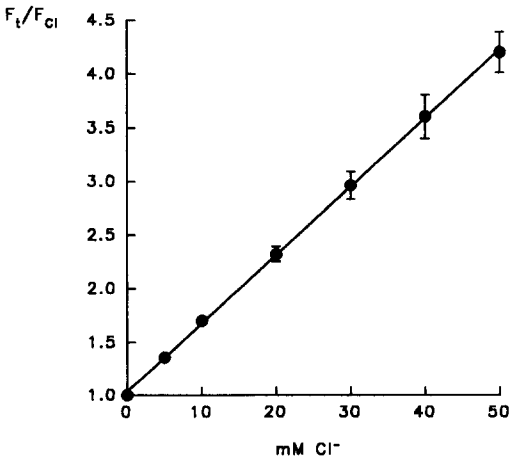

Fig. 3. Calibration of intrasynaptosomal Cl^- versus MQAE. The fluorescence in the absence of chloride divided by the fluorescence in the presence of chloride (F_t/F_{Cl}) is plotted against the Cl^- concentration. The closed symbols (mean of 3–7 experiments ±S.E. per Cl^- concentration) represent the calibration plot. Bars are not shown where the width of the bar does not exceed the width of the symbol. A regression line was fitted to the data, the correlation coefficient (r) was 0.99983.

TABLE I

The mean F_t / F_{Cl} values for five different preparations of synaptoneurosomes and the corresponding intrasynaptoneurosomal chloride concentrations

Synaptoneurosome preparation	Mean $F_t / F_{Cl} \pm$S.E.	mM Chloride [a]
1 ($n = 5$)	2.08±0.12	17.0
2 ($n = 3$)	1.70±0.09	11.0
3 ($n = 4$)	1.61±0.02	9.6
4 ($n = 6$)	1.78±0.11	12.3
5 ($n = 3$)	2.28±0.07	20.1
Mean values±S.E.	1.89±0.25	14.0±3.9

[a] Derived from the standard curve in Fig. 3, calculated according to the Stern–Volmer equation.

tions of pentobarbitone was used to calculate the corresponding chloride concentration according to the Stern–Volmer equation with the above obtained K_{SV} constant (15.7 mM) and the result was plotted into a dose–response curve (Fig. 5). The dose–response relationship shows that the EC_{50} for pentobarbitone was about 35 μM, which corresponds to an outflow of about 300 μM Cl^- from the synaptoneurosomes and a corresponding decrease in the intrasynaptoneurosomal free chloride concentration.

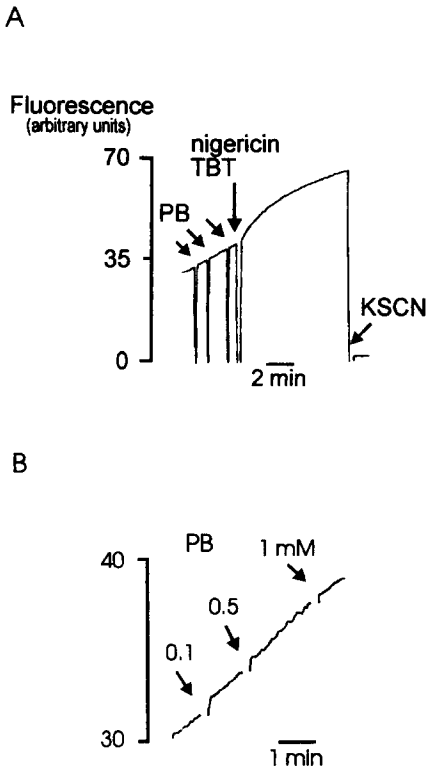

Fig. 4. Effect of the $GABA_A$ receptor agonist, the barbiturate pentobarbitone on the intrasynaptoneurosomal fluorescence of MQAE. Experimental conditions as in Fig. 1. Increasing concentrations of pentobarbitone (PB) were added to synaptoneurosomes in a nominally Cl^- free medium. In B the additions of pentobarbitone are enlarged. Nigericin and tributyltin (TBT) were subsequently added to obtain F_{max}, and KSCN was added to obtain F_t. The basal leakage of Cl^-, before and between additions of PB, is reduced when PB-induced changes in intrasynaptoneurosomal chloride concentration are determined.

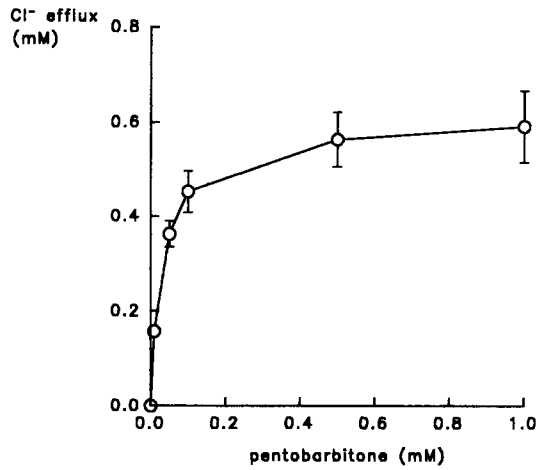

Fig. 5. Dose-dependence of the effect of pentobarbitone on the intrasynaptoneurosomal free Cl^- concentration. Each symbol represents the mean of 3–12 experiments ±S.E. Experimental conditions were as in Fig. 4.

Discussion

The methods traditionally used for measuring transmembrane Cl^- transports are the use of radiolabelled tracers and ion-selective microelectrodes. The advantages of ion-selective microelectrodes are the possibility of simultaneous measurement of ion activities, membrane potential, and electrical membrane properties. The disadvantage is that the method is invasive, it does not measure unidirectional fluxes, and it is not possible to study cell populations. The methods utilising radiolabelled chloride as a tracer measure the total amount of intracellular chloride, not only the free concentration. The use of chloride-sensitive fluorescent indicators enables continuous monitoring of the intracellular free chloride concentration and unidirectional transmembrane chloride transport in single cells or cell populations [15,17,20].

The relationship between the reciprocal of the intracellular MQAE intensity and chloride concentration was linear at least up to 50 mM Cl^-, as has been reported for SPQ [20]. The intrasynaptoneurosomal Stern–Volmer constant for quenching of MQAE by chloride was calculated to 15.7 mM Cl^-. The calculated intracellular K_{SV} for quenching of SPQ by chloride in rabbit proximal tubules [20] was about 83 mM. This indicates that MQAE is intracellularly about five times more sensitive to chloride than SPQ and is therefore more useful when small changes in intracellular free Cl^- concentrations are measured.

The free intracellular chloride concentration in synaptoneurosomes has not been determined before, although synaptoneurosome preparations have frequently been used as experimental models for neurones in several studies on chloride transport [11–14,18]. The intrasynaptoneurosomal free chloride concentration was estimated to be 14 ± 3.9 mM in this study. This corresponds to an activity of 10.5 ± 2.9 mM, approximated with an activity coefficient for NaCl on 0.749 (0.15 M, 38°C). The free intracellular chloride activity in most cells has traditionally been estimated to be about 10 mM or higher [26] in agreement with the results presented here. These values are also fairly close to the K_{SV} constant obtained for quenching of MQAE with Cl^- in this study. Therefore the properties of this indicator are nearly optimal for determining changes in intracellular Cl^-.

The experiments with the $GABA_A$ receptor agonist pentobarbitone induced Cl^- fluxes indicate that it is possible to quantitatively determine the Cl^- fluxes with this fluorescent method. The EC_{50} value for pentobarbitone-induced Cl^- efflux was about 35 μM. This value is somewhat lower than the values obtained in earlier studies, 200 μM with $^{36}Cl^-$ [11,13] and 150 μM with SPQ [16], but fairly close to values obtained for pentobarbitone displacement of [^{35}S]TBPS binding. In this assay IC_{50} values of 53 μM [27] and 95 μM [28] have been obtained. The actual changes in intracellular Cl^- (maximal change around 600 μM Cl^-) seem small when considering that a main portion of central neurones express GABA receptors. The reason for the small fluxes may be due to events such as desensitization, which would prevent gross changes in intracellular Cl^- during inhibitory transmission. It should be noted that desensitization is not seen with this method, because unidirectional fluxes are determined as the Cl^- flowing out will be diluted in the external medium.

In conclusion, MQAE offers a possibility for quantitative determination of the intracellular free chloride activity and transmembrane chloride transport. In studies on $GABA_A$ receptor coupled Cl^- channels with this method, calibration of fluorescence changes can be performed in the same experiment using the ionophores tributyltin and nigericin in combination with SCN^-.

Acknowledgements

This work was supported by grants from the Swedish Culture Foundation, the Swedish Scientific Central Council, the Sigrid Juselius Foundation, the Academy of Finland and the Borg Foundation.

References

1 Alvarez-Leefmans, F.J., Gamino, S.M., Giraldez, F. and Nogueron, I. (1988) J. Physiol. 406, 225–246.
2 Thompson, S.M., Deisz, R.A. and Prince, D.A. (1988) J. Neurophysiol. 60, 105–124.
3 Boron, W.F. (1983) J. Membr. Biol. 72, 1–16.
4 Hoffman, E.K. (1986) Biochim. Biophys. Acta 864, 1–31.
5 Francolini, F. and Petris, A. (1990) Biochim. Biophys. Acta 1031, 247–259.
6 Wagner, J.A., Cozens, A.L., Schulman, H., Gruenert, D.C., Stryer, L. and Gardner, P. (1991) Nature 349, 793–796.
7 Owen, D.G., Segal, M. and Barker, J.L. (1984) Nature 311, 567–570.
8 Jentsch, T.J., Steinmeyer, K. and Schwarz, G. (1990) Nature 348, 510–514.
9 Paulmichl, M., Li, Y., Wickman, K., Ackerman, M., Peralta, E. and Clapham, D. (1992) Nature 356, 238–241.
10 Inoue, M., Hara, M., Zeng, X.-T., Hirose, T., Ohnishi, S., Yasukura, T., Uriu, T., Omori, K., Minato, A. and Inagaki, C. (1991) Neurosci. Lett. 134, 75–78.
11 Schwartz, R.D., Jackson, J.A., Weigert, D., Skolnick, P. and Paul, S.M. (1985) J. Neurosci. 5, 2963–2970.
12 Suzdak, P.D., Schwartz, R.D., Skolnick, P. and Paul, S.M. (1986) Proc. Natl. Acad. Sci. USA 83, 4071–4075.
13 Morrow, A.L., Suzdak, P.D., Karanian, J.W. and Paul, S.M. (1988) J. Pharmacol. Exp. Ther. 246, 158–164.
14 Morrow, A.L., Montpied, P., Lingford-Hughes, A. and Paul, S.M. (1990) Alcohol 7, 237–244.
15 Engblom, A.C., Holopainen, I. and Åkerman, K.E.O. (1989) Neurosci. Lett. 104, 326–330.
16 Engblom, A.C. and Åkerman, K.E.O. (1990) Appl. Fluor. Technol. 2, 8–12.
17 Engblom, A.C., Holopainen, I. and Åkerman, K.E.O. (1991) Brain Res. 568, 55–60.
18 Engblom, A.C. and Åkerman, K.E.O. (1991) J. Neurochem. 57, 384–390.
19 Illsley, N.P. and Verkman, A.S. (1987) Biochemistry 26, 1215–1219.
20 Krapf, R., Berry, C.A. and Verkman, A.S. (1988) Biophys. J. 53, 955–962.
21 Verkman, A.S., Sellers, M.C., Chao, A.C., Leung, T. and Ketcham, R. (1989) Anal. Biochem. 178, 355–361.
22 Hollingsworth, E.B., McNeal, E.T., Burton, J.L., Williams R.J., Daly, J.W. and Creveling, C.R. (1985) J. Neurosci. 5, 2240–2253.
23 Heinonen, E., Åkerman, K.E.O. and Panula, P. (1989) Brain Res. 496, 187–196.
24 Eidelman, O. and Cabantchik, Z.I. (1989) Biochim. Biophys. Acta 988, 319–334.
25 Selwyn, M.J., Dawson, A.P., Stockdale, M. and Gains, N. (1970) Eur. J. Biochem. 14, 120–126.
26 Walker, J.L. and Bang, H.M. (1977) Physiol. Rev. 57, 729–778.
27 Meyers, M.B. and Komiskey, H.L. (1985) Brain Res. 343, 262–267.
28 Cross, A.J., Stirling, J.M., Robinson, T.N., Bowen, D.M., Francis, P.T. and Green, A.R. (1989) Br. J. Pharmacol. 98, 284–290.