Biochimica et Biophysica Acta 940 (1988) 241–246
Elsevier

BBA 73991

Measurement of the cytosolic sodium ion concentration in rat brain synaptosomes by a fluorescence method

Sathapana Kongsamut and Daniel A. Nachshen *

Department of Physiology, Cornell University Medical College, New York, NY (U.S.A.)

(Received 2 September 1987)
(Revised manuscript received 7 December 1987)

Key words: Sodium ion, intracellular; Calcium ion, intracellular; Fluorescence; pH; Ionophore

A method for the measurement of the cytosolic Na^+ concentration in intact synaptosomes is described. This method makes use of a pH sensitive dye (BCECF) that can be loaded into the cytosol and a relatively specific ionophore (monensin) that can exchange Na^+ for H^+ across the synaptosomal membrane. By setting conditions such that there is no electrochemical potential difference for H^+ across the membrane (no membrane potential and $pH_i = pH_o$), addition of ionophore would induce a H^+ flux only if there is a concentration difference for Na^+. Thus, when there is no fluorescence change (no cytosolic pH change) extracellular $[Na^+]$ equals intrasynaptosomal $[Na^+]$. The intrasynaptosomal $[Na^+]$ concentration was determined to be 7 ± 3 mM ($n = 5$; mean $\pm$ S.E.). The results obtained with this fluorescence method are compared with estimates obtained by atomic absorption spectrometry. Limitations and applications of the method are discussed.

Introduction

The Na^+ gradient across cell membranes is vital to many cellular processes. Its regulation by the Na^+/K^+-ATPase has been and continues to be intensively studied (see Refs. 1 and 2). Voltage-sensitive sodium channels are important in cellular excitability where the Na^+ gradient acts as a battery (see Ref. 3). The gradient is also used to drive the transport of substances across the cell membrane: the transport of glucose [4], of amino acids [5] and of neurotransmitters [6] is coupled to the influx of Na^+. Similarly, the efflux of H^+ or of Ca^{2+} is coupled to influx of Na^+. Sodium/hydrogen exchange is important for pH and volume regulation [7]. Sodium/calcium exchange is important in regulating intracellular Ca^{2+} levels [8–11]. In fact, reversal of sodium/calcium exchange during depolarization can provide Ca^{2+} for transmitter release and muscle contraction [11–13]. Because of the importance of the Na^+ gradient in, among other things, Ca^{2+} regulation in synaptosomes, it was desirable to measure intrasynaptosomal $[Na^+]$.

Intracellular $[Na^+]$, $[Na^+]_i$, has generally not been directly measured. It is often assumed that $[Na^+]_i$ is in the low millimolar range as has been measured in the squid giant axon (see Ref. 14). This is perhaps because current methods available for measuring $[Na^+]_i$ are either inaccurate or technically difficult and expensive. Atomic absorption measurements provide an estimate of sodium levels inside cells [15,16] but there is a

* Deceased December 16, 1986.

Correspondence to (present address): S. Kongsamut, Department of Physiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.

large uncertainty inherent to this method due to the many measurements and correction factors involved (see below). Na^+-selective electrodes are only suitable for larger cells and cannot be used in smaller structures such as nerve endings (see Refs. 17–19). We therefore sought to develop a non-invasive method to measure the $[Na^+]_i$ in synaptosomes. The method we describe makes use of a pH-sensitive dye that can be introduced into the cytosol as a permeant ester (BCECF/AM) [20,21] and a selective ionophore that can exchange Na^+ for H^+ (monensin) [28]. We discuss its relevance to other cell systems and how these measurements fit in with previous work on Ca^{2+}, neurotransmitter release and the role of Na^+ in regulating Ca^{2+} levels.

Materials and Methods

Materials. Sprague-Dawley rats were purchased from Hilltop (Scottdale, PA). Percoll was from Pharmacia (Uppsala, Sweden). ^3H_2O and ^{14}C-dextran were from New England Nuclear (Boston, MA). Bis(3,5,5-trimethylhexyl) phthalate (dinonyl phthalate) and dibutyl phthalate were from Fluka AG (Hauppauge, NY). Standard Na^+ and K^+ solutions for atomic absorption measurements were from Alfa Products (Danvers, MA). 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester (BCECF/AM) and quin2/AM were from Molecular Probes (Junction City, OR). Ouabain was obtained from ICN Biomedicals (Irvine, CA). Monensin, nigericin and valinomycin were from Calbiochem-Behring (La Jolla, CA). Gramicidin and veratridine were from Sigma (St. Louis, MO).

Synaptosome preparation. Rat brain synaptosomes were prepared by a modification of two previously described methods [23,24]. Briefly, rats were decapitated and their brains (minus cerebellum and brain stem) were gently homogenized in a solution of 0.32 M sucrose, 5 mM Hepes, 0.1 mM EDTA (pH 7.3), on ice using a loose fitting teflon/glass homogenizer (16 up/down strokes with a 30 s cooling period after 8 strokes). The homogenate was spun at $1000 \times g$ (Sorvall RC-5B, SS-34 rotor, 3000 rpm) for 10 min. The supernatant was then spun at $12000 \times g$ for 20 min to produce a pellet consisting mostly of synaptosomes (white) and mitochondria (brown). The white fluffy part of this pellet was resuspended in 7% (v/v) Percoll in 0.32 M sucrose, 5 mM Hepes and 0.1 mM EDTA (pH 7.4) and layered onto a discontinuous Percoll gradient: 7% on top (9 ml), 10% (15 ml) and 21% (10 ml), prepared in a 50 ml centrifuge tube. This gradient was spun down using the slow start option in a swinging bucket rotor at $15000 \times g$ (Sorvall HB-4; 10500 rpm) for 30 min. The band containing the synaptosomes at the interface between 10% and 21% Percoll was removed and spun down twice in Na solution containing 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, 0.2 mM NaH_2PO_4, 0.2 mM Na sodium pyruvate and 0.005 mM diethylenetriamine pentaacetic acid (DEEA, a heavy metal ion chelator), pH 7.45, to remove sucrose and Percoll. The pellet from the second spin was resuspended and used for the experiments. An advantage of this Percoll gradient over sucrose gradients (see for example, Ref. 25) is that synaptosomes are kept in isosmotic solutions throughout. This synaptosome suspension contains 90–95% presynaptic nerve endings, recognized under electron microscopy as sealed membrane structures containing synaptic vesicles [23,24].

Estimation of sodium and potassium by atomic absorption spectrometry. Amounts of Na^+ and K^+ were measured by atomic absorption spectrometry using a Perkin-Elmer model 360. Total Na^+ and K^+ in intact synaptosomes was subtracted from bound Na^+ and K^+ in lysed synaptosomes. The result was divided by an estimate of intrasynaptosomal volume to obtain $[Na^+]_i$ and $[K^+]_i$ (see Ref. 15). Synaptosomes were prepared as described above and the final pellet (from one rat brain) was resuspended in 3 ml of Na solution with 1 mM $CaCl_2$ (Na + Ca solution), and allowed to equilibrate at 30 °C for 30 min. Aliquots of 150 μl of this suspension were spun down in 1.5 ml of Na + Ca solution in a Beckman microfuge for 90 s and resuspended in 200 μl of various solutions as follows. Suspensions of intact synaptosomes were resuspended in Na + Ca solution and layered on 1 ml phthalate oil (2 : 1 by vol. dibutyl phthalate/dinonyl phthalate) in a 1.5 ml microfuge tube. Other aliquots were lysed with 10 μM digitonin in water and mixed in 1 ml of 10

μM digitonin and not phthalate oil. Some aliquots of both intact and lysed synaptosomes were prepared with ^3H_2O (1.25 μCi/ml final) and ^{14}C-dextran (0.125 μCi/ml final) to label total and extracellular water volumes, respectively. It was thus possible to estimate intrasynaptosomal water volume and to make a correction for any sodium and potassium trapped extracellularly in intact samples. All samples were spun down at $6000 \times g$ for 10 min, the supernatants were carefully suctioned off and the pellet was resuspended in 2 mg/ml LiCl in 2% HCl for atomic absorption readings. Standard curves for Na^+ and K^+ were constructed for each experiment. Each experiment was done in triplicate or quadruplicate. In some experiments, ouabain (1 mM) was added to an aliquot of the synaptosome preparation at the start of the 30 min equilibration period; or BCECF/AM (up to 5 μM) was loaded as described below with parallel vehicle (DMSO) controls. Protein was measured by the method of Lowry et al. [26] using bovine serum albumin as standard.

Fluorescence method for determining sodium. In the fluorescence method, we made use of a fluorescent pH-sensitive dye and of a relatively specific ionophore that can exchange Na^+ for H^+ across synaptosomal plasma membranes. The final synaptosome pellet (from one rat) was resuspended in 4 ml of Na solution and loaded with BCECF/AM (0.2 μM) for 30 min at 30 °C. This acetoxymethyl (AM) ester is freely permeable across the synaptosomal membrane and once inside is cleaved by cytosolic esterases to yield and trap the charged, pH sensitive free acid [20,21]. After loading, the synaptosome suspension was twice diluted (into approx. 40 ml of Na solution) and spun down ($6000 \times g$, 5 min). The second pellet was resuspended in 4 ml Na + Ca solution and allowed to incubate at 30 °C for 30 min.

Following incubation, a measurement of intrasynaptosomal pH was made (see Fig. 1). This was done by taking an aliquot of the synaptosome suspension, spinning it down (in 1.5 ml of Na + Ca solution in a Beckman microfuge for 90 s) and resuspending in 2.0 ml of Na + Ca solution in a cuvette. Fluorescence was recorded using a Farrand System 3 Spectrofluorometer with excitation and emission wavelengths set at 500 and 530 nm, respectively, as previously described [27]. The cuvette was surrounded by a jacket of circulating water maintained at 30 °C. At this relatively low loading concentration, all BCECF/AM had usually de-esterified by this time. Some additional de-esterification was seen, as an increasing fluorescence signal, when higher loading concentrations were used. When a constant signal was obtained, nigericin (0.67 μM final) and gramicidin A (1 μM final) were added to make the membrane permeable to H^+ ions, which would make pH_i equal to pH_o (pH 7.45). This treatment presumably also made the membrane permeable to Na^+ and K^+ and abolished their concentration differences. The pH was then adjusted downward to the original fluorescence level (pH_i) with 0.1 M HCl and this was read with a pH meter. All subsequent solutions were adjusted to this pH. In other samples, an appropriate volume of 0.1 M HCl was added (to make pH_o equal pH_i) at the same time as nigericin and gramicidin. Fluorescence, after an initial transient, did not change. This was an additional check on the accuracy of the pH measurement.

The $[Na^+]_i$ measurement, performed with the remainder of the BCECF-loaded synaptosomes entailed adjusting extracellular conditions as follows: 100 mM KCl, x mM NaCl, $(50 - x)$ mM *N*-Methyl-D-glucamine (NMGCl), 10 mM glucose, 10 mM Hepes, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 0.2 mM sodium pyruvate and 0.005 mM DEEA (pH adjusted to pH_i for the particular experiment). This mixture was prepared from stock solutions of Na solution, NMGCl solution (where NaCl was replaced by NMGCl), and KCl solution (where NaCl was replaced by KCl). Thus all stock solutions were isomolar. Aliquots (200 μl) of the synaptosome suspension were spun down in a Beckman microfuge as described and resuspended in $2000x/145$ μl of Na + Ca solution where x was the desired mM Na concentration. This volume was added to the cuvette containing the other constituents (such that total volume was 2 ml), already positioned in the spectrofluorometer (with stir bar spinning), and immediately (within 5 s) monensin (10 μ/ml) was added while fluorescence was continuously recorded on a chart recorder at high speed. The first few seconds recorded established a baseline. Any fluorescence

changes following addition of monensin were noted. An increase in fluorescence indicated that $[Na^+]_o$ was greater than $[Na^+]_i$ while a decrease in fluorescence indicated that $[Na^+]_o$ was less than $[Na^+]_i$. The concentration at which fluorescence did not change was taken as the point where $[Na^+]_o$ equals $[Na^+]_i$. Monensin is a carboxylic ionophore that predominantly exchanges Na^+ for H^+ across membranes [22,28]. In some experiments, 0.5 mM amiloride, 3 μM tetradotoxin and 0.1 mM D-600 were added to block Na^+/H^+ exchange, sodium channels and calcium channels, respectively. There was no obvious difference between the absence and presence of these drugs.

Results

The Percoll method for preparing synaptosomes maintains them under isosmotic conditions and hence they would not undergo any of the changes that accompany volume stress (see, for example, Refs. 7, 23 and 29). As seen in Table I, the intrasynaptosomal volume (5.00 ± 0.36 ml/g protein, mean $\pm$ S.E., $n = 17$ experiments in triplicate or quadruplicate) is greater than that reported by Blaustein and Goldring [15] (4.05 ± 0.14 ml/g protein; $n = 4$), who used sucrose gradient-prepared synaptosomes. The intrasynaptosomal $[K^+]$, measured by atomic absorption spectrometry, is similar to that reported by Blaustein and Goldring [15]. They reported a $[K^+]_i$ of 100 mM, compared with our estimate of 97 ± 23 mM. Our atomic absorption measurement of $[Na^+]_i$ (2 ± 11 mM) is much lower than the value obtained by Blaustein and Goldring of 19 mM and may reflect differences in the synaptosome preparation since volume stressed synaptosomes would have elevated Na^+/H^+ exchange activity so that $[Na^+]_i$ should be elevated. Ouabain, an inhibitor of the Na^+/K^+-ATPase enzyme, would be expected to and did raise $[Na^+]_i$ (to 43 ± 18 mM). BCECF-loaded synaptosomes did not have a significantly different sodium concentration from control. It should be noted that this atomic absorption method of measuring cation concentrations is subject to large variations (note the range of values obtained obtained for each measurement; Table I) primarily as a result of the many individual measurements and correction factors involved.

The fluorescence method is more direct and gives more consistent results. It uses intact (live) synaptosomes and should come closer to measuring $[Na^+]_i$ in synaptosomes. Fig. 2 shows a typical measurement of $[Na^+]_i$. Synaptosomes were prepared and pH_i was measured (Fig. 1) as described. The experiment then consisted of varying the $[Na^+]_o$ until a null point was reached where no change in fluorescence was recorded (in this case between 5 and 10 mM), at which point $[Na^+]_o = [Na^+]_i$. Similar experiments were performed in synaptosomes treated with ouabain (Na^+/K^+ in-

TABLE I

INTRASYNAPTOSOMAL SODIUM AND POTASSIUM MEASUREMENTS WITH THE ATOMIC ABSORPTION METHOD

Values are means $\pm$ S.E.; values in brackets are the range of values obtained in n experiments. $[Na]_i = 2 \pm 11$ mM; 43 ± 18 mM in ouabain; 5 ± 18 mM in BCECF. $[K]_i = 97 \pm 23$ mM.

	Na (mM)	n	K (mM)	n	H_2O
Intact	39 ± 5 (0–89)	17	106 ± 18 (71–106)	4	5.0 ± 0.4 (μl/mg protein) $n = 17$
Ouabain (1 mM)	79 ± 12 (50–116)	5	–		
BCECF (5 μM loaded)	42 ± 12 (0–77)	6	–		
Lysed	37 ± 6 (8–89)	13	9 ± 5	4	

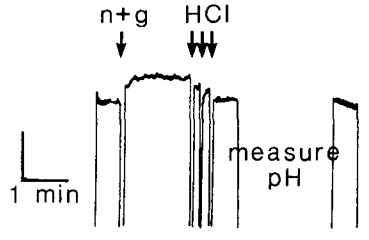

Fig. 1. Measurement of intrasynaptosomal pH. Synaptosomes loaded with BCECF were placed in a cuvette and fluorescence recorded as shown. After the reading had stabilized, nigericin (n) and gramicidin (g) (final concentrations 0.67 μM and 1 μM, respectively) were added to permeabilize the membrane to H^+ and after the reading restabilized at pH_o (7.45), HCl (0.1 M) was added to bring pH down to pH_i and this value was measured. Average pH_i was found to be 7.04 ± 0.3; $n = 14$. See text for details.

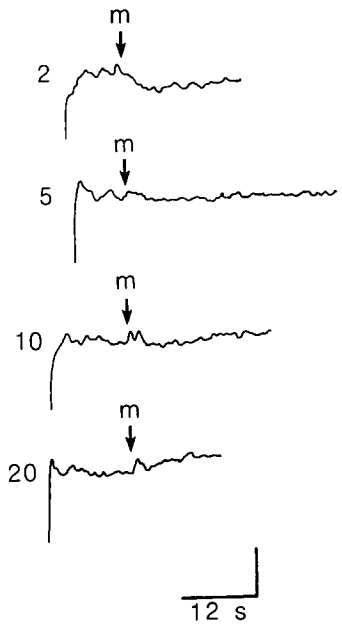

Fig. 2. Measurement of intrasynaptosomal sodium. After determination of pH_i (see Fig. 1), all solutions were adjusted to this pH. Synaptosomes were placed in solutions of various sodium concentrations (as labelled in mM), monensin was added (m, final concentration 10 μg/ml; at arrow) and fluorescence (pH) changes were noted. The trace where no fluorescence change could be detected, in this case 5 mM, was taken as the trace where $Na_o = Na_i$. The smallest detectable fluorescence change was about 0.1 units on the chart recorder which represents a fluorescence change of about 1.5%. See text for details.

hibitor) and veratridine (which causes a persistent Na^+ influx by holding sodium channels open [3]). Both treatments raised $[Na^+]_i$ (see Table II). Interestingly, at high BCECF concentrations (2.5 to 5 μM BCECF/AM loading) $[Na^+]_i$ was increased to 80 mM. This was not due to formaldehyde released from the de-esterification reaction as similar concentrations of quin2/AM loaded in a similar manner did not affect measured Na^+ concentration (Table II). Removal of extracellular Ca^{2+} caused $[Na^+]_i$ to increase to 50 mM; this could be partially reversed when Ca^{2+} was added back.

TABLE II

SUMMARY OF Na_i MEASUREMENTS WITH THE FLUORESCENCE METHOD

Values are means ± S.E.; values in brackets are the range of values obtained in *n* experiments.

	Na (mM)	*n*
Control (0.2 μM BCECF)	7 ± 3 (2–15)	5
Ouabain (1 mM)	50–70	1
Veratridine (10 μM)	80	1
BCECF/AM (5 μM loaded)	80	3
Quin2/AM (5 μM loaded)	5	2
No added calcium	50 ± 5 (40–80)	6
Calcium added back	39 ± 3 (20–50)	6

Potassium measurements using the same principle were attempted with the potassium-selective ionophores nigericin (0.67 μM) or valinomycin (0.5 μM). $[Na^+]_o$ was held constant at 10 mM and $[K^+]_o$ and $[NMG^+]_o$ were varied such that they added up to 140 mM or more. However, all attempts yielded impossible results ($K_i > 300$ mM).

Discussion

We have introduced a new method for measuring intracellular sodium using simple principles and available tools. The method is technically easy and uses intact, viable synaptosomes. We see no reason why this method cannot be applied to cells or to other membrane systems into which BCECF can be loaded. The principle of the method is not entirely new; a null point method has been used to measure $[Ca^{2+}]_i$ using arsenazo III as the Ca^{2+} indicator and digitonin to permeabilize liver cells [30]. The major limitation we see is that the method lacks time resolution; it cannot be used to follow changes in $[Na^+]_i$ on a time scale of seconds or even minutes. However, knowledge of steady state $[Na^+]_i$ is often valuable. As discussed in the Introduction, the trans-plasma membrane Na^+ gradient is used to regulate and transport many ions and molecules.

The physiological basis for making these measurements was our interest in the regulation of intrasynaptosomal calcium as it pertains to transmitter release [31,32]. The regulation of $[Ca^{2+}]_i$ in nerve terminals is central to its main function of releasing neurotransmitters since a rise in $[Ca^{2+}]_i$ is prerequisite to release (see Ref. 33). The regulation of Ca^{2+} in synaptosomes is primarily by the Na^+/Ca^{2+} exchanger [11,34]. Because the exchanger has a $Na^+ : Ca^{2+}$ ratio of 3 : 1, a small change in $[Na^+]_i$ can profoundly affect Ca^{2+} ef-

flux. The present measurements show that $[Na^+]_i$ in synaptosomes is much lower than previously thought. In addition, they show that Na^+ influx through the Na^+/Ca^{2+} exchanger can be larger than Na^+ efflux through the Na^+/K^+-ATPase. Importantly, when Ca^{2+} was omitted from the external medium, $[Na^+]_i$ increased to very high levels (> 50 mM) and this was partially reversible when Ca^{2+} was reintroduced into the medium. Our $[Na^+]_i$ measurements compare well with measurements made by Lee et al. [17] in heart cells using Na^+ selective electrodes. They obtained $[Na^+]_i$ values of 6 to 8 mM. In addition, they found that lowering of extracellular calcium concentration from 2.7 mM to 1.08 mM produced an increase in $[Na^+]_i$ from 5.7 mM to 9.2 mM [17].

During the sodium measurements, we found that using high loading concentrations of BCECF/AM caused higher concentrations of sodium to be measured. We believe that this was an artefact of the method and that sodium concentrations did not actually become this high (80 mM). We know that it was not caused by de-esterification releasing formaldehyde since quin2/AM loaded in the same manner did not cause a high concentration to be measured. In addition, 5 μM BCECF/AM loading did not raise $[Na^+]_i$ as measured by atomic absorption spectrometry. However, users of this method should be aware of this potential problem which may or may not occur in other systems.

We hope that the method presented here with its advantages and limitations will find some use in physiological studies. We have used this method to show that in synaptosomes, Na^+/Ca^{2+} exchange can operate faster than Na^+/K^+-ATPase. Although the dogma of low intracellular sodium probably holds for most cell types, knowledge of the precise value of the sodium gradient is often useful.

Acknowledgements

The authors would like to thank Drs. Pierre Drapeau, Sergio Sanchez-Armass and Olaf Andersen for valuable comments and suggestions. This was supported by USPHS grant NS 20464 and by an investigatorship from the New York Heart Association to D.A.N. D.A.N. was a Cornell Scholar in Biomedical Sciences.

References

1 Skou, J.C. (1965) Physiol. Rev. 45, 596–617.
2 Cantley, L. (1986) Trends Neural Sci. 9, 1–3.
3 Catterall, W.A. (1984) Science 233, 653–661.
4 Hopfer, U. and Groseclose, R. (1980) J. Biol. Chem. 255, 4453–4462.
5 Wingrove, T.G. and Kimmich, G.A. (1985) Ann. N.Y. Acad. Sci. 456, 80–82.
6 Kanner, B.I. (1983) Biochim. Biophys. Acta 726, 293–316.
7 Grinstein, S. and Rothstein, A. (1986) J. Membr. Biol. 90, 1–12.
8 Langer, G.A. (1982) Annu. Rev. Physiol. 44, 435–449.
9 Eisner, D.A. and Lederer, W.J. (1985) Am. J. Physiol. 248, C189–C202.
10 Reeves, J. (1986) Curr. Top. Membr. Transp. 25, 77–127.
11 Nachshen, D.A. (1988) J. Physiol. (London), submitted.
12 Coutinho, O.P., Carvalho, C.A.M. and Carvalho, A.P. (1984) Brain Res. 290, 261–271.
13 Sheu, S.-S., Sharma, V.K. and Uglesity, A. (1986) Am. J. Physiol. 250, C651–C656.
14 Hodgkin, A.L. (1958) Proc. Roy. Soc. (London) Ser. B 148, 1–37.
15 Blaustein, M.P. and Goldring, J.M. (1975) J. Physiol. (London) 247, 589–615.
16 Owen, N.E. and Villereal, M.L. (1985) Am. J. Physiol. 248, C288–C295.
17 Lee, C.O., Kang, D.H., Sokol, J.H. and Lee, K.S. (1980) Biophys. J. 29, 315–330.
18 Moody, G.J. (1985) J. Biomed. Eng. 7, 183–195.
19 Tsien, R.Y. (1983) Annu. Rev. Biophys. Bioeng. 12, 91–116.
20 Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell. Biol. 95, 189–196.
21 Paradiso, A.M., Tsien, R.Y. and Machen, T.E. (1984) Proc. Natl. Acad. Sci. USA 81, 7436–7440.
22 Choy, E.M., Evans, D.F. and Cussler, E.L. (1974) J. Am. Chem. Soc. 96, 7085–7090.
23 Nagy, A. and Delgado-Escueta, A.V. (1984) J. Neurochem. 43, 1114–1123.
24 Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kidd, G.J. and Rostas, J.A.P. (1986) Brain Res. 372, 115–129.
25 Hajos, F. (1975) Brain Res. 93, 485–489.
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 19, 255–265.
27 Nachshen, D.A. (1987) J. Physiol. 387, 415–423.
28 Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530.
29 Parker, J.C. and Castranova, V. (1984) J. Gen. Physiol. 84, 379–401.
30 Murphy, E., Coll, K., Rich, T.L. and Williamson, J.R. (1980) J. Biol. Chem. 255, 6600–6608.
31 Nachshen, D.A., Sanchez-Armass, S. and Weinstein, A.M. (1986) J. Physiol. (London) 381, 17–28.
32 Nachshen, D.A. (1988) in The Presynaptic Regulation of Neurotransmitter Release (Feigenbaum, J.J. and Hanani, M., eds.), in press, Freund Publishing, London.
33 Katz, B. (1971) Science 173, 123–126.
34 Nachshen, D.A. (1985) J. Physiol. (London) 363, 87–101.