Biochimica et Biophysica Acta, 1107 (1992) 175–178

BBAMEM 75634

The Na^+–Ca^{2+} exchanger activity in cerebrocortical nerve endings is reduced in old compared to young and mature rats when it operates as a Ca^{2+} influx or efflux pathway

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(Received 23 January 1992)

Key words: Sodium–calcium ion exchanger; Voltage sensitivity; Calcium ion channel; Intracytosolic calcium ion; Calcium ion, intracytosolic; Aging; Cerebrocortical nerve ending

The activity of the Na^+–Ca^{2+} exchanger, which regulates the entry and the extrusion of Ca^{2+} ions from nerve endings was investigated in Percoll-purified cerebrocortical synaptosomes of aged rats. $^{45}Ca^{2+}$ uptake in a Na^+-free medium and $^{45}Ca^{2+}$ efflux in a 145 mM Na^+ medium were significantly reduced in cerebrocortical synaptosomes from aged rats (24 months) as compared to those occurring in young (4 months) and mature (14 months) rats. $^{45}Ca^{2+}$ influx induced by 55 mM K^+, a concentration of K^+ ions which selectively promotes Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (VSCC), was significantly reduced in mature and aged rats as compared to that occurring in young rats. The impairment of these mechanisms in aged rats is not accompanied by any variation of fura-2 monitored Ca^{2+} levels under resting and depolarizing conditions.

Introduction

Voltage-sensitive calcium channels (VSCC), the bidirectional Na^+–Ca^{2+} exchanger and the Ca^{2+}-Mg^{2+}-ATPase are the main plasma membrane mechanisms responsible for both sudden intracellular Ca^{2+} elevation and subsequent return to resting Ca^{2+} concentrations [1].

It has been recently suggested that the impairment of Ca^{2+} homeostasis in neuronal cells may be the triggering event which leads to the development of brain aging [2,3]. Although a large number of studies have been performed in order to demonstrate an impairment of the plasma membrane mechanisms involved in the mantainance of Ca^{2+} levels both in resting or in stimulated conditions [3–6], the relative role played by the Na^+–Ca^{2+} exchanger and VSCC in the alteration of Ca^{2+} regulation during brain aging is not clearly established [5,6].

The aim of the present study was to evaluate the possible impairment of Ca^{2+} ion entry and extrusion in cerebrocortical nerve endings of aged rats.

For this purpose appropriate extracellular ionic conditions and time intervals, which selectively activate VSCC or the Na^+–Ca^{2+} exchanger when this antiporter operates as a Ca^{2+} influx or efflux pathway, were chosen [7,8].

The results of the present study showed that in cerebrocortical nerve endings of aged rats there is a marked reduction of the activity of the Na^+–Ca^{2+} exchanger in the forward and reverse mode of action. In addition the entrance of Ca^{2+} ions through VSCC is also reduced in mature and aged rats.

Methods

Synaptosomal preparation

Synaptosomal fractions from the brain of male Sprague Dawley rats of different age (4, 14 and 24 months) were obtained according to the procedure of Dunkley et al. [9] using a discontinuous Percoll gradient.

Briefly, rats were killed and brains removed and kept on ice. The cortex was dissected and homogenized in 10 vol of ice-cold sucrose medium, whose composi-

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tion was (in mM): 320 sucrose, 1 EDTA, 0.25 DTT, adjusted to pH 7.4.

$^{45}Ca^{2+}$ influx determinations

Resuspended synaptosomes were incubated in 145 mM Na^+ for 30 min at 37°C to allow the Na^+ accumulation into the nerve terminals. After this period, Ca^{2+} uptake was initiated by injecting 0.225 ml of different solutions (55 mM K^+ or 145 mM choline) containing 4.9 μM $^{45}Ca^{2+}$ into a 25 μl synaptosome suspension (200 μg of proteins) and terminated at different times (1, 3, 30 s) by injecting 0.450 ml of 20 mM EGTA ice-cold quench solution. After three rinses, each with 2.5 ml of 1 mM EGTA wash solution, synaptosomes were immediately filtered through Whatman fiber glass GFC filters. Filters were then placed into scintillation vials with 5 ml of scintillation cocktail (Dynagel, Baker) and the trapped radioactivity was determined by liquid scintillation counting.

$^{45}Ca^{2+}$ efflux studies

After a 60 min preincubation period in a 145 mM Na^+-containing solution, synaptosomes were loaded for 15 s with 10 μM $^{45}Ca^{2+}$ in presence of 75 mM K^+ in a medium containing (mM): 75 NaCl, 75 KCl, 1.2 $MgCl_2$, 10 Hepes and 10 glucose (pH 7.4 at 37°C) in a final incubation volume of 200 μl. $^{45}Ca^{2+}$ uptake was terminated and $^{45}Ca^{2+}$ efflux was initiated simultaneously by the addition to the tubes of 4.5 ml of a $^{45}Ca^{2+}$ free prewarmed (37°C) medium containing either 145 mM choline or 145 mM NaCl. At 60 s, synaptosomes were filtered and filters were washed and counted as previously described. $^{45}Ca^{2+}$ efflux was calculated as the difference between $^{45}Ca^{2+}$ content retained in synaptosomes (time 0) and that after 60 s efflux time.

Determination of intrasynaptosomal Ca^{2+} levels

Resuspended synaptosomes (3 mg protein/ml) were equilibrated for 10 min at 37°C in a Ca^{2+}-free medium. After this period, synaptosomes were exposed to 5 μM fura-2/AM for 30 min in presence of 0.5% bovine serum albumin. After the loading, synaptosomes were diluted 10-fold and spun at 11000 × *g* for 10 min to remove the extracellular dye. The synaptosomal pellet was resuspended at a protein concentration of 6 mg/ml in a Ca^{2+}-free medium and kept on ice. Before each experiment, 100 μl of loaded synaptosomes were centrifuged (9500 × *g* for 3 min) and the resulting pellet was resuspended in 2 ml of Ca^{2+} free medium at 37°C and added to a thermostated cuvette in a Perkin-Elmer LS-S spectrofluorimeter equipped with a magnetic stirrer. 1 mM Ca^{2+} was added to synaptosomes 30 s after the beginning of the experiment. Fluorescence intensity of fura-2 was recorded at an excitation and emission wavelength of 340 and 490 nm, respectively.

Calibration of the fluorescent signal was performed according to the method of Grynkiewicz et al. [10].

Protein determination

Synaptosomal protein concentration was determined by the method described by Bradford [11].

Statistics

All experiments were done at least three times. Results were expressed as means ± S.E. Statistical analysis of the data was performed by means of analysis of variance, followed by Neuman-Keul's test.

Results

55 mM K^+-induced $^{45}Ca^{2+}$ influx in cerebrocortical synaptosomes of young, mature and aged rats

In order to evaluate $^{45}Ca^{2+}$ entrance through VSCC, cerebrocortical synaptosomes were exposed to 55 mM extracellular K^+ ions, a concentration which selectively promotes Ca^{2+} entrance through VSCC without the activation of the Na^+–Ca^{2+} exchanger when this antiporter operates as a Ca^{2+} influx pathway [7]. $^{45}Ca^{2+}$ influx was evaluated at very short time intervals (1 and 3 s), which are more closely related to the activation-inactivation kinetics of VSCC [12].

The results showed that 55 mM K^+-induced $^{45}Ca^{2+}$ influx was significantly reduced in synaptosomes from mature (14 months) and aged (24 months) animals as compared to young (4 months) rats. This reduction in 14- and 24-month-old rats was observed at all the considered time intervals (1, 3 and 30 s) (Fig. 1A). On the other hand, $^{45}Ca^{2+}$ uptake which occurs during a 30-s interval also depends on the activation of VSCC, since the ionic concentration of K^+ ions chosen in these experiments (55 mM K^+) does not promote Ca^{2+} entrance through the Na^+–Ca^{2+} exchanger [7].

$^{45}Ca^{2+}$ influx and efflux through the Na^+–Ca^{2+} exchanger in cerebrocortical synaptosomes

In order to study the activity of the Na^+–Ca^{2+} antiporter when this exchange system operates as a Ca^{2+} influx pathway, cerebrocortical synaptosomes

TABLE I

Intrasynaptosomal Ca^{2+} levels in cerebrocortical synaptosomes from 4-, 14- and 24-month-old rats

The data are the mean ± S.E. of at least seven experiments.

Age	$[Ca^{2+}]_i$ (nM); ionic conditions	
	5 mM K^+	35 mM K^+
4 months	374 ± 29	678 ± 58
14 months	333 ± 17	830 ± 58
24 months	356 ± 21	734 ± 69

were incubated in a Na^+-free medium (containing 145 mM choline), an ionic condition in which $^{45}Ca^{2+}$ entry depends only on the activity of the Na^+–Ca^{2+} antiporter [7]. In this ionic condition $^{45}Ca^{2+}$ uptake was significantly lower in aged animals than that observed in young and mature rats (Fig. 1B).

When cerebrocortical synaptosomes, loaded with $^{45}Ca^{2+}$ in presence of depolarizing concentrations of K^+ ions, are incubated in an extracellular medium containing 145 mM Na^+ ions for 60 s, an inwardly directed Na^+ gradient is generated which favours the operation of the Na^+–Ca^{2+} antiporter as a Ca^{2+} efflux pathway. Data showed that in cerebrocortical synaptosomes from aged rats $^{45}Ca^{2+}$ efflux was significantly reduced as compared to that occurring in synaptosomes from young and mature rats (Fig. 1C).

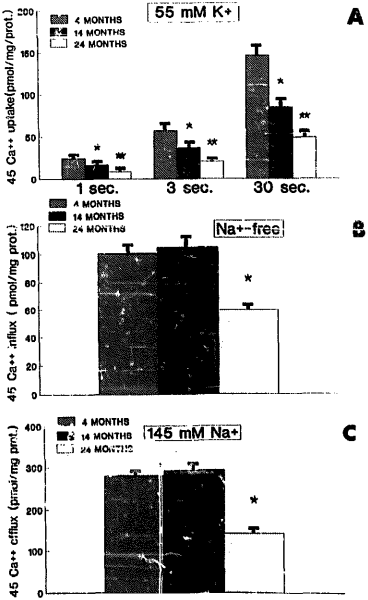

Fig. 1. $^{45}Ca^{2+}$ influx evoked by 55 mM K^+ (A), Na^+-dependent $^{45}Ca^{2+}$ influx (B) and $^{45}Ca^{2+}$ efflux (C) in cerebrocortical synaptosomes from young, mature and aged rats. (A) Cerebrocortical synaptosomes from young (4 months), mature (14 months) and aged (24 months) rats, preincubated in a 145 mM Na^+ medium for 30 min, were then exposed to a depolarizing stimulus (55 mM K^+) for various time intervals (1, 3 and 30 s) in presence of 4.9 μM $^{45}Ca^{2+}$. Each basal $^{45}Ca^{2+}$ uptake value obtained in 145 mM Na^+ has been subtracted from the respective 55 mM K^+-elicited Ca^{2+} uptake value. (B) Cerebrocortical synaptosomes from young (4 months), mature (14 months) and aged (24 months) rats, preincubated for 30 min in 145 mM Na^+ containing medium, were exposed to a Na^+-free medium (145 mM choline) in presence of 4.9 μM $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ taken up in normal solution (145 mM Na^+) has been subtracted from the values shown. (C) Synaptosomes preincubated for 60 min in a 145 mM Na^+ solution, were loaded for 15 s with 10 μM $^{45}Ca^{2+}$ in a 75 mM K^+ containing medium. $^{45}Ca^{2+}$ efflux was initiated (time 0) by dilution of the loading medium with 4.5 ml of $^{45}Ca^{2+}$-free medium containing either 145 mM choline or 145 mM Na^+. $^{45}Ca^{2+}$ efflux was stopped after 60 s with wash and quench solutions. $^{45}Ca^{2+}$ efflux values were obtained from the difference between $^{45}Ca^{2+}$ content retained in synaptosomes after efflux time (60 s) and that at time 0. Each basal $^{45}Ca^{2+}$ efflux value obtained in 145 mM choline was subtracted from the respective 145 mM Na^+-elicited Ca^{2+} efflux value. Data are the mean ± S.E. of at least three separate experiments in which at least three rats at each age were used. In each experiment at least four separate determinations were carried out. (A) * $P < 0.05$ vs. young and aged rats, ** $P < 0.05$ vs. young and mature rats. (B) * $P < 0.05$ vs. young and mature rats. (C) * $P < 0.05$ vs. young and mature rats.

Intracytosolic Ca^{2+} levels in resting and after 35 mM K^+-induced depolarization in cerebrocortical synaptosomes from young, mature and aged rats

Cytosolic Ca^{2+} levels in Percoll-purified cerebrocortical synaptosomes in aged (24 months) rats were not different from those monitored in young (4 months) and mature (14 months) rats either in resting or in depolarizing (35 mM K^+) conditions (Table I).

Discussion

The results of the present study showed that in the nerve endings of cerebrocortical neurons, a reduction of the activity of the bidirectional Na^+–Ca^{2+} exchanger occurs in 24-month-old rats, whereas a decrease of $^{45}Ca^{2+}$ uptake through VSCC was found in mature and old rats. The impairment of these mechanisms, which play a relevant role in the entrance and extrusion of Ca^{2+} ions [1,13], is not accompanied in nerve endings of aged rats by any variation of fura-2 monitored $[Ca^{2+}]_i$ levels in resting and depolarizing conditions.

The reduced activity of VSCC in aged rats, observed in the present study during the fast phase, confirms the observation of Leslie et al. [14], who found that the decline of Ca^{2+} entrance can be more appropriately detected at very short time intervals (1–3 s) which are more closely related to the half-life of opening and

subsequent inactivation of Ca^{2+} channels [12]. A reduction of $^{45}Ca^{2+}$ influx through VSCC has also been reported when radiotracer ion fluxes were measured at time intervals longer than 3 s [4,5]. In addition, the reduced activity of VSCC observed in the present study and in the mentioned reports does not seem to be due to changes in membrane potential [4]. Furthermore, the results of the present work showed a reduced capacity of the Na^+–Ca^{2+} exchanger when it acts in the physiological direction to extrude Ca^{2+} ions from cerebrocortical nerve endings of aged rats. Since this exchange system can also operate in the opposite way [13], allowing Ca^{2+} ions to enter into the neurons, the antiporter activity was investigated by evaluating $^{45}Ca^{2+}$ influx in a non depolarizing medium (Na^+-free). As expected, a decrease of $^{45}Ca^{2+}$ uptake was observed. In accordance with this finding, Martinez et al. [4] found that when the Na^+–Ca^{2+} exchanger is activated by increases of membrane potential higher than −20 mV, $^{45}Ca^{2+}$ uptake is also reduced in synaptosomes from aged rats. Since in the present study the reduction of Na^+-dependent $^{45}Ca^{2+}$ uptake was detected in slightly hyperpolarized nerve endings [15], it can be concluded that the Na^+–Ca^{2+} exchanger displays a reduced activity during aging regardless of the state of the membrane potential. This reduction seems to be the consequence of a reduced affinity of the antiporter for Ca^{2+} ions when it operates as a Ca^{2+} influx pathway [16]. During the aging process the Na^+–Ca^{2+} exchanger is not the only membrane extrusion system that is impaired, in fact the V_{max} for Ca^{2+} activation of the Ca^{2+}-activated Mg^{2+}-dependent pump is also reduced [16]. Finally, the evaluation of intrasynaptosomal Ca^{2+} levels performed in the present study and other investigations [17,18] failed to show any significant variation in the cation concentration both in resting conditions and after depolarization. The possible difference between the data obtained in $^{45}Ca^{2+}$ uptake and fura-2-monitored Ca^{2+} levels in cortical synaptosomes from mature and aged rats after K^+-induced depolarization can be explained by the different methodological procedures. In fact, studies performed with the radiotracer allow to detect only cation entering through VSCC during fast phase (1 s), whereas during fluorimetric experiments, fura-2 monitored intrasynaptosomal Ca^{2+} levels represent the balance between the activity of intracellular Ca^{2+} buffering systems and Ca^{2+} influx and efflux mechanisms.

Acknowledgements

We wish to thank Fidia Research Laboratories, Abano Terme, Italy, for providing us young, mature and aged animals used in the present paper. This work was supported in part by 40% and 60% M.U.R.S.T. Grants and C.N.R. Grants No. 90.04055.CT04 and No. 90.01279-CT14 to L.A. and G.F.D.R. The excellent technical assistance of Mr. Vincenzo Grillo is gratefully acknowledged.

References

1 Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
2 Gibson, G.E. and Peterson, C. (1987) Neurobiol. Aging 8, 329–343.
3 Roth, G.S. (1989) Ann. NY Acad. Sci, 568, 68–73.
4 Martinez, A., Vitórica, J., Bogónez, E. and Satrústegui, J. (1987) Brain Res. 435, 249–24.
5 Peterson, C. and Gibson, G.E. (1983) J. Biol. Chem. 19, 11482–11483.
6 Peterson, C., Nicholls, D.G. and Gibson, G.E. (1985) Neurobiol Aging 6, 297–304.
7 Taglialatela, M., Di Renzo, G.F. and Annunziato, L. (1990) Mol. Pharmacol. 38, 385–392.
8 Taglialatela, M., Canzoniero, L.M.T., Cragoe, E.J., Jr., Di Renzo, G.F. and Annunziato, L. (1990) Mol. Pharmacol. 38, 393–400.
9 Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kidd, G.J. and Rostas, J.A.P. (1986) Brain Res. 372, 115–129.
10 Grynkiewicz, G., Poenie, M. and Tsien, Y. (1985) J. Biol. Chem. 260, 3440–3450.
11 Bradford, M.M.A. (1976) Anal. Biochem. 72, 248–254.
12 Sushiw, J.B., Leary, M.E. and Murawsky, M.M. (1986) J. Neurosci. 6, 1349–1357.
13 Sanchez-Armass, S. and Blaustein, M. (1987) Am. J. Physiol. 252, C595–C603.
14 Leslie, S.W., Chandler, L.J., Barr, E.M. and Farrar, R.F. (1985) Brain Res. 329, 177–183.
15 Taglialatela, M., Canzoniero, L.M.T., Fatatis, A., Di Renzo, G.F., Yasumoto, T. and Annunziato, L. (1990) Biochim. Biophys. Acta 1026, 126–132.
16 Michaelis, M.L., Johe, K. and Kitos, T.E. (1984) Mech. Ageing Dev. 53, 392–398.
17 Giovannelli, L. and Pepeu, G. (1989) J. Neurochem. 53, 392–398.
18 Farrar, R.P., Rezazadeh, S.M., Morris, J.L., Dildy, J.E., Gnau, K. and Leslie, S.W. (1989) Neurosci. Lett. 100, 319–325.