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EFFECT OF AGING ON CALCIUM CYTOSOLIC CONCENTRATION IN RAT CORTICAL SYNAPTOSOMES

G. PEPEU and L. GIOVANNELLI

Dipartimento di Farmacologia Preclinica e Clinica, Università degli Studi, Florence, Italy

Summary. - *Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured in synaptosomes prepared from the cerebral cortex of 3, 16 and 24 month-old male Charles River Wistar rats. Electron microscopy examination demonstrated no morphological differences between the synaptosomes prepared from 3 and 24 month-old rats. Age did not modify $[Ca^{2+}]_i$, as measured by the QUIN 2 technique, both at rest and immediately after depolarization with 50 mM K^+. The Ca^{2+} load following depolarization was cleared in about 13 min in the 3 month-old rats. The rate of clearance was significantly slower both in the 16 ($p < 0.01$) and in the 24 month-old ($p < 0.0001$). A prolonged calcium influx may be responsible for the slower clearance of Ca^{2+} load in aged rats.*

Riassunto (Effetto dell'età sulla concentrazione di calcio citosolico in sinaptosomi di corteccia di ratto). - *Le concentrazioni citosoliche di Ca^{2+} ($[Ca^{2+}]_i$) sono state misurate in sinaptosomi preparati dalla corteccia cerebrale di ratti maschi Wistar Charles River di 3, 16 e 24 mesi di età. L'esame al microscopio elettronico non ha messo in evidenza differenze morfologiche fra i sinaptosomi di ratti di 3 e 24 mesi. Non sono state osservate modificazioni indotte dall'età del $[Ca^{2+}]_i$, misurato mediante la tecnica del QUIN 2, né a riposo né immediatamente dopo depolarizzazione causata dall'aggiunta di K^+ 50 mM. L'aumento della concentrazione di Ca^{2+} indotto dalla depolarizzazione ritornava ai valori di riposo in circa 13 min nei ratti di 3 mesi mentre nei ratti di 16 e 24 mesi la velocità di allontanamento del calcio era significativamente ridotta ($p < 0.01$ e $p < 0.0001$, rispettivamente). Si ritiene che il prolungamento del tempo di influsso del Ca^{2+} possa essere la causa del rallentato ritorno del $[Ca^{2+}]_i$ ai livelli di riposo.*

Introduction

Aging is associated with the reduction of cognitive and non cognitive nervous functions resulting from specific neuronal alterations and losses [1-17] and an ensuing impairment in neurotrasmission. The latter deficit largely depends on a decrease in neurotransmitter synthesis and release [6-8]. Its causes are still a matter of investigations and changes in membrane fluidity [11] and reduction in energy metabolism [12] have been taken into consideration. Transient increases in cytosolic ionized calcium concentrations, in response to many stimuli, act as a trigger for neurotransmitter release [5] and it has been suggested [7] that alterations of the mechanisms regulating calcium homeostasis, occurring in the aging brain, may be responsible for some age-dependent changes of the neuronal cell function including the decrease in neurotransmitter release.

The aim of the present work has been to study the effect of age on cytosolic calcium concentration, using the fluorescent probe QUIN 2 [26] in cortical synaptosomes at rest and after depolarization.

Methods

Synaptosome preparation

Male Charles River rats 3, 16 and 24 months old, were killed by decapitation and the brain rapidly removed and placed in ice-cold physiological solution. The cortex was then dissected and homogenized in 10 vol of ice-cold 0.32 M sucrose. Purified synaptosomes were prepared according to the method of Hayos [10].

The conditions and the contamination of the purified synaptosomal preparation were checked by electron microscopy examination. Specimens prepared from 3 and 24 month-old rats were fixed in glutaraldehyde in 0.1 cacodylate buffer, pH 7.4, at 5 °C for 3 h followed by postfixation in 1% OsO_4. They were then dehydrated in acetone series, passed through propylene oxide and embedded in Epon 812. Ultrathin sections were stained with uranylacetate and alkaline bismuth-subnitrate and examined with a Siemens Elmiskop 102 electron microscope at 80 kV.

QUIN 2 loading and fluorescence measurements

A suspension of purified synaptosomes, with a protein concentration of 2 mg/ml, was prepared in a pre-warmed (37 °C) oxygenated incubation medium having the following composition (mM): NaCl 125, KCl 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 0.02, $NaHCO_3$ 5, HEPES 25 (pH 7.4), glucose 6, bovine serum albumine (BSA) 0.08. After 15 min preincubation at 37 °C, aliquots of a 10 mM solution of QUIN 2-AM in dimethyl sulphoxide (DMSO) were added to the resuspended synaptosomes, giving a final dye concentration of 20 - 30 μM. The incubation was allowed to proceed for 15 min, then the suspension was diluted 10 folds with pre-warmed, oxygenated incubation medium without BSA. After further 30 min incubation, the suspension was centrifuged at 6000 *g* for 15 min (0 °C temperature) and the pellet resuspended in ice-cold Ca^{2+}- and BSA-free medium at the concentration of 2 mg protein/ml. The suspension was kept on ice for more than 3 hours. Immediately before each measurement, aliquots (1 ml) of the suspension were again centrifuged and resuspended in 1.5 ml of pre-warmed and oxygenated medium (1 mM $CaCl_2$) without BSA, giving a final protein concentration of about 1.3 mg/ml. The suspension was then transferred to a quartz fluorescence cuvette continuosly stirred at 37 °C, and the readings were made in a Perkin Elmer 650-10S spectrofluorimeter using excitation and emission wavelengths of 339 (2 nm slit) and 492 (10 nm slit), respectively.

All the measurements were done after equilibrating the suspension for 5 min in the cuvette. Emission spectra were recorded in order to check the hydrolysis of the dye by the spectral shift from 430 (emission peak of QUIN 2-AM) to 492 nm (emission peak of QUIN 2). The hydrolysis was usually complete. The addition of TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) - ethylenediamine), a permeant heavy metal chelator [2], did not modify the signal in samples prepared from either adult or aged animals. Drugs were added directly into the cuvette in small aliquots from concentrated stock solutions.

$[Ca^{2+}]_i$ values were obtained from the equation:

$$[Ca^{2+}] = 115 \text{ nM } \frac{(F - F_{min})}{(F_{max} - F)}$$

[26]. The maximal (F_{max}) and minimal (F_{min}) indicator fluorescences were obtained following the calibration procedure described by Meldolesi *et al.* [16] which also makes it possible to evaluate the leakage of the dye out of the synaptosomes into the medium. The leakage was subtracted from the total. The percentage of the QUIN 2 content leaking out from the synaptosomes was 0.67 ± 0.05 ($n = 9$) per min in preparations from the 3, 0.80 ± 0.08 ($n = 4$) from the 16 and 0.57 ± 0.02 ($n = 6$) from the 24 month-old rats, respectively. The differences are not statistically significant indicating that age did not affect membrane permeability.

At the end of each experiment, the fluorescence of non-loaded synaptosomes (i.e. synaptosomes exposed to DMSO only during the loading procedure and then treated as the loaded ones) was measured, and the drugs used during the experiment were tested again in order to evaluate autofluorescence changes.

The intraterminal concentration of the dye was calculated by comparing the F_{max} of the samples with the F_{max} of known amounts of QUIN 2 added to lysed, unloaded synaptosomes. Under the described experimental conditions it was approximately 0.4 mM.

Protein determination

The protein concentration of each sample was determined with the method of Lowry *et al.* [14 bis] using bovine serum albumine as a standard.

Chemicals

Standard laboratory chemicals were obtained from Merck (Darmstadt, FRG). QUIN 2 free acid, QUIN 2-AM, TPEN, EGTA, DMSO and verapamil were supplied from Sigma Chemical Co. (St. Louis, MO 63178, USA). $^{45}CaCl_2$ (specific activity 10 mCi/mg calcium) was purchased from Amersham International (Little Chalfont Buckinghamshire, England), and Instagel from Packard Instruments Co. (Downers Grove, IL 60515, USA).

Statistical analysis

The statistical analysis of the data obtained in the Ca^{2+} uptake experiments has been carried out by the Student's t-test between two means. The differences in $[Ca^{2+}]_i$ at rest and after depolarization between adult and aged animals in fluorescence experiments have been analyze by ANOVA. Analysis of coefficient of variance (ANOCOV) has been used to analyze the rate of clearing of the Ca^{2+} load induced by depolarization in the three groups of animals, taking as independent variables the time and the age.

Results

Series of electron micrographs of the purified synaptosomes prepared from 3 and 24 month-old rats were taken through the whole thickness of the pellets. No morphological and quantitative differences between young and old rats were observed. The counting of the different types of particles revealed the presence of about 15% of mitochondria, and contamination by other particles was even smaller. The majority of the particles was represented by pre-and post-synaptic membrane specializations and neurotransmitter vesicles.

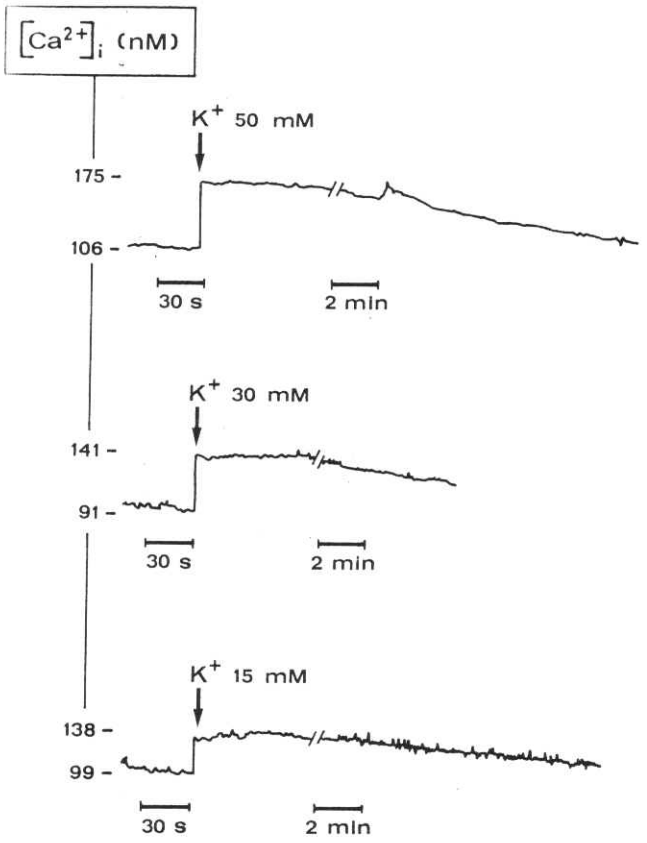

Fig. 1. - Fluorescence traces of synaptosomes loaded with QUIN-2. Increase in cytosolic $[Ca^{2+}]_i$ brought about by 50, 30 and 15 mM K^+ depolarization. The figures on the left side represent $[Ca^{2+}]_i$ in nM. The time scale is indicated below each trace.

Fig. 1 shows the fluorescence traces of the cytosolic calcium concentrations at rest and after depolarization induced by different K^+ concentrations added to the incubating medium. The traces represent the fluorescence of loaded synaptosomes. Upon depolarization, synaptosomal $[Ca^{2+}]_i$ rapidly increases and remains at the peak level for 30-60 s. Then it begins to return to the resting level. Fig. 2 shows the linear relationship between $(K^+)_e$ and the increase in $[Ca^{2+}]_i$.

The addition of verapamil at concentrations of 20, 40 and 60 μM, before depolarization, brought about a reduction of 16, 43 and 67% respectively, of the increase in $[Ca^{2+}]_i$ elicited by depolarization. When added 1 min after depolarization, 60 μM of verapamil markedly accelerated the decline of $[Ca^{2+}]_i$. Concentrations of verapamil of 20 and 40 μM have a proportionally lesser effect.

Fig. 3 shows the time course of $[Ca^{2+}]_i$ changes induced by depolarization in synaptosomes prepared from rats of different ages. $[Ca^{2+}]_i$ is slightly higher in 24 than in 16 and 3 month-old rats both at rest and at peak after depolarization, although the differences between the 3 groups are not statistically significant. However, there is a significant difference in the time course of the return to resting level. Cytosolic Ca^{2+} levels remain more elevated in 24 than in the 16 and 3 month-old rats during 13 min following depolarization. These results do not de-

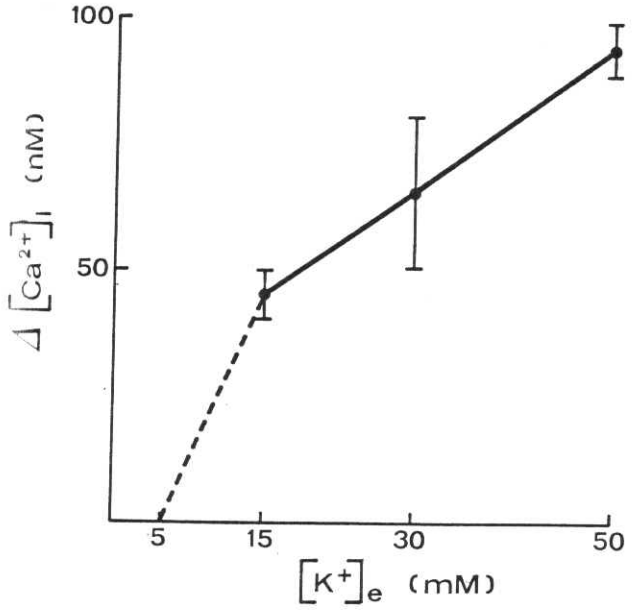

Fig. 2. - Linear relationship between depolarizing $[K^+]_e$ concentration in mM and the increase in cytosolic $[Ca^{2+}]_i$ in nM. Each point is the mean of 4 experiments. Vertical bars: SEM of the mean.

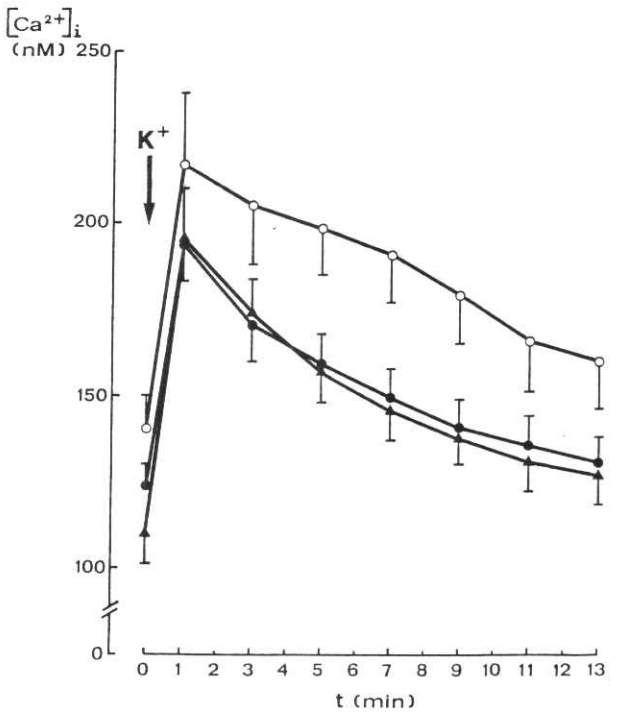

Fig. 3. - Effect of depolarization on $[Ca^{2+}]_i$ values in 3 (closed circles), 16 (triangles) and 24 (open circles) month-old rats. At the time indicated with 0 50 mM K^+ was added. Each point is the mean ± SEM from at least 4 animals.

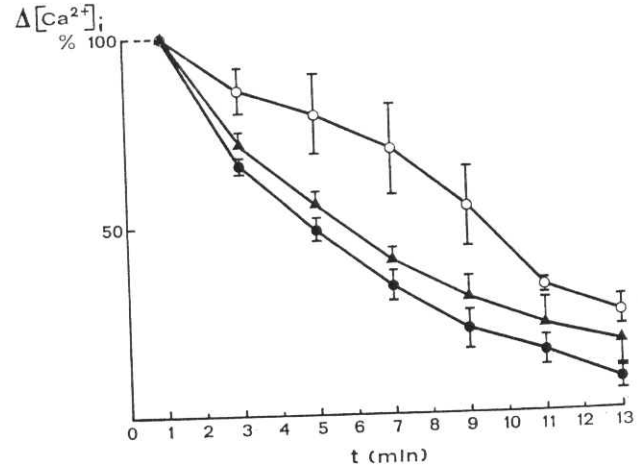

Fig. 4. - Temporal decay of $[Ca^{2+}]_i$ increase ($\Delta[Ca^{2+}]_i$) upon depolarization in 3 (closed circles), 16 (triangles) and 24 (open circles) month-old rats. The values are expressed as percent (mean of at least 4 animals ± SEM) of the difference between $[Ca^{2+}]_i$ measured 1 min after depolarization and the resting $[Ca^{2+}]_i$ level. Statistical analysis of the curves carried out by ANOCOV has demonstrated that the differences between the 3 and the 16 month/old rats are statistically significant with $p < 0.01$, those between the 3 and the 24 month/old with $p < 0.0001$, and those between the 16 and the 24 month/old with $p < 0.001$.

pend on the difference in resting levels because they can also be seen when $[Ca^{2+}]_i$ levels are expressed as percent of the peak increase induced by depolarization, as shown in Fig. 4. The rate at which synaptosomes prepared from aged brains clear the Ca^{2+} load brought about by depolarization, is lower than in younger animals. The delay exists already in 16 but it is much more evident in 24 month-old rats. The statistical analysis of the three curves has demonstrated that the differences between the values measured at different times are statistically significant in the three groups ($p < 0.0001$). The differences between the groups are also statistically significant either comparing the 3 month-old with the 16 month -old rats ($F = 7.035$; $df_1 = 1$; $df_2 = 112$), or the 3 month-old with the 24 month-old ($F = 56.117$; $df_1 = 1$, $df_2 = 106$), or the 16 month-old with the 24 month-old ($F = 25.596$, $df_1 = 1$, $df_2 = 52$).

Discussion

According to Martinez *et al.* [15] Ca^{2+} concentration in depolarized synaptosomes, estimated by the QUIN 2 method is higher in 24 than in 3 month-old rats, and indirect evidence that age may increase intracellular calcium concentration is offered by electrophysiological studies on hippocampal neurons [13]. In the present experiments no significant changes of cytosolic $[Ca^{2+}]_i$ at rest and at peak after depolarization were detected. However, a delay in the return of cytosolic calcium concentration to the resting level, after the increase brought about by K^+ depolarization, was found. This indicates an age-induced change in the mechanisms regulating calcium homeostasis in cortical synaptosomes.

An age-dependent decrease in K^+ depolarization-induced Ca^{2+} uptake from cortical synaptosomes, restricted to the fast Ca^{2+} influx has been also observed [14-22]. The possibility has been suggested [14] that the reduction in the fast phase of voltage-dependent Ca^{2+} uptake, involving type L and N calcium channels, may be responsible for the age-induced decrease in evoked neurotransmitter release from the brain, which has been demonstrated in both cortical slices [21] and synaptosomes [18].

The lack of difference, found in our experiments, in peak cytosolic $[Ca^{2+}]_i$ between young and aging rats is only apparently at variance with the decrease in calcium uptake in aging rats and may be explained by the buffering power of the cytosol [19] and, to some extent, of the fluorescent dye and the limited resolution time of the fluorescence experiments, which makes it difficult to reveal Ca^{2+} transients.

Our experiments demonstrate that a delay in clearing Ca^{2+} after depolarization occurs in synaptosomes prepared from aging rats.

The Na^+/Ca^{2+} exchange is considered an important mechanism in allowing nerve terminals to recover from Ca^{2+} loading [20-23]. It has been shown [15] that the operation of the Na^+/Ca^{2+} exchange, evaluated in total brain synaptosomes by removing Na^+ from the incubation medium, is reduced in aging rats. In our experiments, the addition, after depolarization, of the Ca^{2+} channel blocker, verapamil, markedly accelerates the return of $[Ca^{2+}]_i$ to the resting level. This finding indicates that the slow decline of cytosolic $[Ca^{2+}]_i$ is a balance between extrusion and sequestration mechanisms, and a long lasting slow influx, presumably due to new Ca^{2+} through activated Ca^{2+} channels. It has been shown that inactivation of presynaptic Ca^{2+} channels during depolarization is slow and incomplete [3] and organic Ca^{2+} antagonists are selective blockers of the slow-inactivating Ca^{2+} channels [25]. An increased influx through L channels in the aging brain is consistent with the observation of an age-dependent increase in verapamil [4] and nitrendipine [9] binding sites in cortical membranes.

The physiological implications of the slow decline in $[Ca^{2+}]_i$ in aging rats are difficult to interpret under the light of the recently demonstrated [24] spatiotemporal dynamics of intracellular calcium concentrations during spontaneous and evoked nervous activity. However, the delay in clearing Ca^{2+} is evidence of the modifications induced by age in the functional properties of neuronal membranes.

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REFERENCES

1. AGNATI, L.F., FUXE, K., BENFENATI, F., TOFFANO, G., CIMINO, M., BATTISTINI, N., CALZÀ, L. & MERLO PICH, E. 1984. Studies on aging processes. *Acta Physiol. Scand. Suppl.* **532**: 45-66.

2. ARSLAN, P., DI VIRGILIO, F., BELTRAME, M., TSIEN, R.Y. & POZZAN, T. 1985. Cytosolic Ca^{2+} homeostasis in Ehrlich and Yoshida carcinomas. *J. Biol. Chem.* **260**: 2719-2727.

3. AUGUSTINE, G.J., CHARLTON, M.P. & SMITH, S.J. 1987. Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* **10**: 633-693.

4. BATTAINI, F., GOVONI, S., RIUS, R.A. & TRABUCCHI, M. 1985. Age-dependent increase in [^{3}H] verapamil binding to rat cortical membranes. *Neurosci. Lett.* **61**: 67-71.

5. CUTHBERT, A.W. 1988. The multiple physiological roles of calcium: possible sites of pharmacological intervention. In: *Calcium in drug actions*. P.F. Baker (Ed.). Springer Verlag, Berlin. pp. 1-6.

6. DOMINO, E.F., DREN, A.T. & GIARDINA, W.J. 1978. Biochemical and neurotransmitter changes in the aging brain. In: *Psychopharmacology: a generation of progress*. A. Di Mascio & K.F. Killam (Eds). Raven Press, New York. pp. 1507-1515.

7. GIBSON, G.E. & PETERSON, C. 1987. Calcium and the aging nervous system. *Neurobiol. Aging* **8**: 329-343.

8. GOTTFRIES, C.G. 1985. Alzheimer's disease and senile dementia: biochemical characteristics and aspect of treatment. *Psychopharmacology* **86**: 245-252.

9. GOVONI, S., RIUS, R.A., BATTAINI, F., BIANCHI, A. & TRABUCCHI, M. 1985. Age related reduced affinity in [^{3}H]nitrendipine labelling of brain voltage-dependent calcium channels. *Brain Res.* **333**: 374-377.

10. HAJOS, F. 1975. An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* **295**: 485-489.

11. HERSHKOVITZ, M. 1983. Mechanisms of brain aging - the role of membrane fluidity. In: *Aging of the brain*. W.H. Gispen & J. Traber (Eds). Elsevier, Amsterdam. pp. 85-99.

12. HOYER, S. 1985. The effect of age on glucose and energy metabolism in brain cortex of rats. *Arch. Gerontol. Geriatr.* **4**: 193-203.

13. LANDFIELD, P.W. & PITLER, T.A. 1984. Prolonged Ca^{2+}-dependent after hyperpolarizations in hippocampal neurons in aged rats. *Science* **226**: 1089-1092.

14. LESLIE, S.W., CHANDLER, L.J., BARR, E.M. & FARRAR, L.P. 1985. Reduced calcium uptake by rat brain mitochondria and synaptosomes in response to aging. *Brain Res.* **329**: 177-183.

14 bis. LOWRY, D.H., ROSEBROUGH, N.J., FARR, L. & RANDALL, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

15. MARTINEZ, A., VITORICA, J., BOGONEZ, E. & SATRUSTEGUI, J. 1987. Differential effects of age on the pathways of calcium influx into nerve terminals. *Brain Res.* **435**: 249-257.

16. MELDOLESI, J., MALGAROLI, A., WOLLHEIM, C.B. & POZZAN, T. 1987. Ca^{2+} transients and secretion: studies with QUIN 2 and other Ca^{2+} indicators. In: In vitro *methods for studying secretion*. G. Potsner & G.M. Trifaro (Eds). Elsevier, Amsterdam. pp. 283-307.

17. MESULAM, M.M., MUFSON, E.J. & ROGERS, J. 1987. Age related shrinkage of cortically projecting cholinergic neurons: a selective effect. *Ann. Neurol.* **22**: 31-36.

18. MEYER, E.M., ONGE, E. ST. & CREWS, F.T. 1984. Effects of aging on rat cortical presynaptic cholinergic processes. *Neurobiol. Aging* **5**: 315-317.

19. NACHSHEN, D.A. 1985. Regulation of cytosolic calcium concentration in presynaptic nerve endings isolated from rat brain. *J. Physiol. (London)* **363**: 87-101.

20. NACHSHEN, D.A., SANCHEZ-ARMASS, S. & WEINSTEIN, A.M. 1986. The regulation of cytosolic calcium in rat brain synaptosomes by sodium-dependent calcium efflux. *J. Physiol. (London)* **381**: 17-28.

21. PEDATA, F., GIOVANNELLI, L., SPIGNOLI, G., GIOVANNINI, M.G. & PEPEU, G. 1985. Phosphatidylserine increases acetylcholine release from cortical slices in aged rats. *Neurobiol. Aging* **6**: 337-339.

22. PETERSON, C. & GIBSON, G.E. 1983. Aging and 3,4-diaminopyridine alter synaptosomal calcium uptake. *J. Biol. Chem.* **258**: 11482-11486.

23. SANCHEZ-ARMASS, S. & BLAUSTEIN, M.P. 1987. Role of sodium-calcium exchange in regulation of intracellular calcium in nerve terminals. *Am. J. Physiol.* **252**: C595-C603.

24. TANK, D.W., SUGIMORI, M., CONNOR, J.A. & LLINAS, R.R. 1988. Spatially resolved calcium dynamics of mammalian Purkinje cells in cerebellar slice. *Science* **242**: 773-777.

25. TRIGGLE, D.J. & JANIS, R.A. 1987. Calcium channel ligands. *Annu. Rev. Pharmacol. Toxicol.* **27**: 347-369.

26. TSIEN, R.Y., POZZAN, T. & RINK, T.J. 1982. Calcium homeosthasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell. Biol.* **94**: 325-334.