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CHANGES IN CYTOSOLIC FREE CALCIUM WITH 1,2,3,4-TETRAHYDRO-5-AMINOACRIDINE, 4-AMINOPYRIDINE AND 3,4-DIAMINOPYRIDINE

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Abstract—The effects of 1,2,3,4-tetrahydro-5-aminoacridine (THA), 4-aminopyridine (4-AP) and 3,4-diaminopyridine (3,4-DAP) on cytosolic free calcium (Ca_i^{2+}) were determined. Both 4-AP and THA have been used to treat Alzheimer's disease. THA is a structural analog of the aminopyridines, which alter calcium homeostasis in nerve terminals. The structural similarities between these compounds suggest a common mechanism of action. The aminopyridines raised Ca_i^{2+} concentrations in non-depolarized synaptosomes, whereas THA had no effect. Neither the aminopyridines nor THA had any effect on Ca_i^{2+} concentrations in potassium-depolarized synaptosomes. These results suggest that the beneficial effects of THA may be mediated by other mechanisms (i.e. neurotransmitter degradative enzyme inhibition), whereas those of 4-AP and 3,4-DAP may be due, at least in part, to their elevation of Ca_i^{2+}, which may enhance neurotransmitter release or other calcium-dependent processes.

Both 4-aminopyridine (4-AP†) [1] and 1,2,3,4-tetrahydro-9-aminoacridine (THA) [2] have been used in the treatment of Alzheimer's disease. 3,4-Diaminopyridine (3,4-DAP), a more neurologically potent, but less toxic aminopyridine, produces effects similar to 4-AP in animal studies [3, 4], but has not yet been examined clinically with Alzheimer's disease. In rats, 3,4-DAP diminishes behavioral deficits due to aging [5–7] or to AF64A, a toxin that is proposed to produce Alzheimer-like neurological deficits in animals [8].

The precise mechanism of action of these compounds is unknown. Although the best studied action of THA is as an acetylcholinesterase inhibitor [9], its structural similarity to the aminopyridines, which are generally regarded to be potassium channel blockers [10], suggests that the clinical efficacy of THA may be mediated in part through an interaction with potassium channels. A direct effect on potassium channels was demonstrated recently with high concentrations of THA [11]. The aminopyridines, either through their actions on potassium channels or by other mechanisms [12], increase calcium uptake in tissues from aged rodents [13] or humans [14] as well as Alzheimer patients [14]. Since there is a close relationship between voltage-dependent potassium channels, intracellular calcium concentrations, and neurotransmitter release, the effects of micromolar concentrations of THA, 4-AP, and 3,4-DAP on cytosolic free calcium (Ca_i^{2+}) using the fluorescent probe fura-2 and mouse forebrain synaptosomes were determined.

MATERIALS AND METHODS

Materials. The fura-2 and the fura-2 AM (FAM) were from Molecular Probes (Eugene, OR). The male CD-1 mice (20–30 g) were from Charles Rivers Inc. (Wilmington, MA). The fluorescence measurements were done with a delta scan from Photon Technology International (Princeton, NJ), which has a heated sample chamber and a magnetic stirrer to maintain a uniform suspension of the sample. Dimethyl sulfoxide (DMSO), *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), 3-[*N*-morpholino]propane sulfonic acid (MOPS) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from the Sigma Chemical Co. (St. Louis, MO). Plastic cuvettes (Spectrocell, Oreland, PA), Triton X-100 (J. T. Baker Chemical Co., Phillipsburg, NJ), $CaCl_2$ standard for fura-2 calibration (Radiometer, Copenhagen) and THA (Aldrich Chemical Co., Milwaukee, WI) were from the indicated companies.

Incubation medium. The incubation medium included the following (pH 7.4; final concentration in mM): $CaCl_2$ (1.3), NaCl (131.0), $MgSO_4$ (1.3), KCl (5), KH_2PO_4 (0.4), Na^+TES (20.0) and glucose (1.5). The high temperature coefficient of the TES necessitated particular care to check the pH at 37°. To depolarize the synaptosomes, KCl in 20 μl of distilled water was injected to give a final concentration of 31 mM.

Tissue preparation. Synaptosomes were prepared from whole mouse brain excluding the cerebellum and olfactory bulbs [15]. Following decapitation, the brain was immediately placed in ice-cold isolation buffer (0.32 M sucrose, 5 mM Na^+HEPES) and weighed. The brain was sliced on a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY)

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† Abbreviations: 4-AP, 4-aminopyridine; THA, 1,2,3,4-tetrahydro-5-aminoacridine or tacrine; 3,4-DAP, 3,4-diaminopyridine; FAM, fura-2 acetoxymethylester; Ca_i^{2+} cytosolic free calcium; DMSO, dimethyl sulfoxide; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; SNK, Student–Newman–Keul's test; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and MOPS, 3-[*N*-morpholino]propane sulfonic acid.

at a 1-mm setting, returned to the isolation medium, and rinsed. The tissue was homogenized in isolation buffer (10% of original weight/volume) in a 10-ml glass homogenizer with twelve strokes of a Teflon pestle. The homogenate was centrifuged at 0° for 5 min at 1000 *g*. The supernatant fraction was transferred to a pretared tube and spun for 12 min at 17,000 *g*. The pellet was gently resuspended in incubation buffer (10% w/v; pH 7.4 at 0°) with a small, loosely fitting Teflon pestle and was then centrifuged for 10 min at 7000 *g*. The pellet was resuspended in cold incubation buffer (pH 7.4, 0°) to a final concentration of 50 mg tissue/ml and stored on ice.

Cytosolic free calcium measurement. Individual samples were incubated with FAM immediately before the fluorescence determination. Aliquots (0.25 ml) of the tissue suspension were added into each of two microfuge tubes. One milliliter of incubation buffer (pH 7.4, 37°) at room temperature was added to each tube and the pair spun in an Eppendorf Microfuge 5414 for 15 sec (15,000 *g*). The supernatant fractions were aspirated, and the pellets resuspended in 1 ml of fresh incubation buffer. The contents of both microfuge tubes (2 ml) were then transferred into a plastic scintillation vial and 4 μl of FAM (1 mM in DMSO) was added. The FAM was stored under nitrogen in a sealed vial with a latex septum and removed with a Hamilton syringe. FAM stored in this manner was stable for prolonged periods, whereas it gradually decomposed when exposed to air. After addition of FAM, the contents of each sample vial were triturated with a plastic pipet. Samples were oxygenated for 30 sec with a flow of 6–8 standard cubic feet per hour of 100% oxygen, capped, and incubated for 20 min at 37° in a shaking water bath.

After incubation, the sample was divided equally between two microfuge tubes and spun for 15 sec. The supernatant fractions were discarded, and the tissue in each tube was rinsed with 1 ml of incubation buffer and spun again. The supernatant fraction was aspirated, and the final pellet was resuspended in 1 ml of incubation buffer that had been maintained at 37°. The contents of the two tubes were transferred into a plastic cuvette containing a micro spin bar. The tissue was evenly suspended in the cuvette by trituration and allowed to equilibrate for 3 min in the delta scan sample chamber at 37°. The sample was then monitored for 90 sec by alternating the excitation wavelengths between 340 and 380 nm twenty times a second; the calculated calcium from these measurements is referred to as the basal value. Thus, when numbers are expressed as percent of basal, they have been divided by this measurement for each sample. The drug was then added at the specified concentration and the sample was monitored for another 90 sec. KCl (final concentration, 31 mM) was added, and the sample was monitored for an additional 90 sec. Triton X-100 (20 μl of 10% w/w) was added to lyse the synaptosomes, and the sample was monitored again for 90 sec. The total time in the chamber was 360 sec. With each sample, a parallel cuvette was run in which the synaptosomes were treated identically except that they were centrifuged instead of adding Triton X-100. The counts per second (CPS) in the supernatant fraction from this centrifugation represented the fura-2–Ca^{2+}-FAM signal external to the tissue.

Tissue autofluorescence was determined by treating a sample exactly as described above except that no FAM was added. Tissue autofluorescence varied with the different treatments (e.g. KCl, Triton vs non-Triton) so that its accurate estimation under each condition was essential. These values were subtracted from each sample.

The signal due to nonhydrolyzed ester was subtracted from the raw sample values to more accurately assess the fura-2 ratio [16, 17]. Since after lysis saturating concentrations of calcium (1.3 mM) were present extracellularly, any fura-2 was complexed to calcium so that only two species were present: the fura-2–calcium complex and the nonhydrolyzed ester. Under these conditions, the 340/380 fluorescence ratio (S_{max}) predicts the mole fraction of the nonhydrolyzed dye:

$$\ln[\text{mole fraction FAM}] = 0.1762 - 1.0465\ S_{max} \quad (r = 0.999)$$

This relation was used to determine the contribution of FAM to the signal from the unlysed preparation as follows. The total CPS at 380 nm after Triton equals the sum of the CPS due to FAM and that due to fura-2:

$$CPS_{380} = CPS_{FAM} + CPS_{FURA} \tag{1}$$

The concentration of each component is its mole fraction times the total number of picomoles/per milliliter of both species. Since the total mole fraction is 1 (i.e. the mole fraction of fura-2 plus the mole fraction of FAM = 1), the mole fraction of fura-2 equals 1 minus the mole fraction of FAM. Thus, if X equals the total number of picomoles per milliliter:

$$\begin{aligned}[\text{FAM}] &= (\text{mole fraction FAM})\ (X)\\ [\text{FURA}] &= (1 - \text{mole fraction of FAM})\ (X)\end{aligned} \tag{2}$$

The CPS due to each compound at 380 nm is its concentration of the compound times its molar fluorescence coefficient (m). Under these conditions the molar extinction coefficient of fura was 12.0 and that of FAM was 117.

$$CPS_{380} = (m_{FAM380})\ (\text{mole fraction of FAM})\ (X) + (m_{Fura380})\ (1 - \text{mole fraction of FAM})\ (X) \tag{3}$$

Since CPS_{380}, m_{FAM380}, and $m_{Fura380}$ and the mole fraction of FAM are all known, the total number of picomoles per milliliter and the picomoles of FAM can be calculated. The contribution of FAM to the signal after excitation at both 340 and 380 nm in the intact synaptosomes can then be calculated. The molar extinction coefficients in the buffer that mimics intracellular buffer [18] were determined to be 58.0 at 380 nm and 13.5 at 340 nm.

$$\begin{aligned}CPS_{FAM380i} &= [m_{FAM380i}]\ [\text{FAM}]\\ CPS_{FAM340i} &= [m_{FAM340i}]\ [\text{FAM}]\end{aligned} \tag{4}$$

The CPS due to FAM can then be subtracted from the corrected CPS signal for the basal and treated sample values at 340 and 380 nm to give the CPS due only to fura-2 in each channel. Once the FAM contribution to the measured signal has been subtracted, cytosolic calcium can be calculated by established relations [18].

$$[Ca_i^{2+}] = (230\,nM)\frac{R - R_{min}}{R_{max} - R} \times \frac{(CPS_{380}\text{ from }R_{min})}{(CPS_{380}\text{ from }R_{max})}$$

The 230 nM is the fura-2–Ca^{2+} dissociation constant [18]. The R_{max} values were from the Triton-treated samples. After correction for the FAM, the R_{min} values were relatively constant.

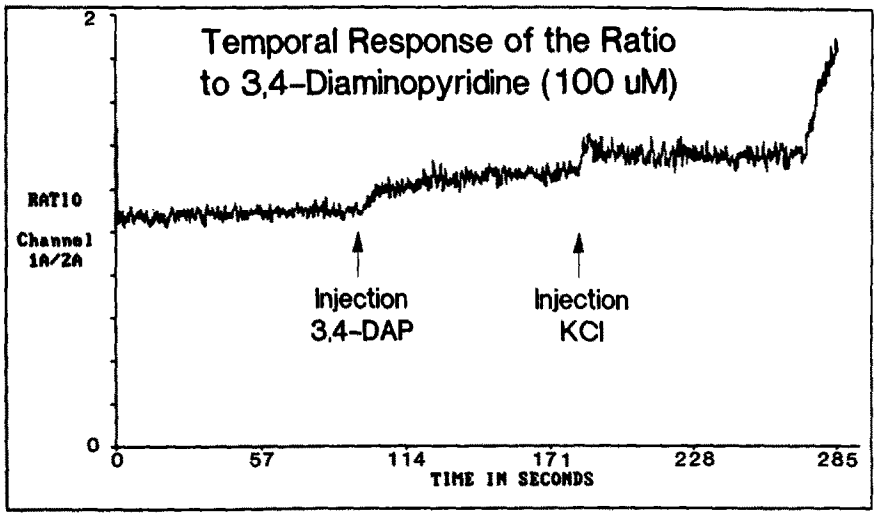
Fluorescent methods are sensitive to compounds that may absorb the fluorescent signal. THA at concentrations of greater than 100 μM quenched the sample so much that it was not possible to correct for the interference. At 10 μM it was possible to correct for a diminution in the signal of about 9%.

All statistical comparisons were by one-way analysis of variance followed by the Student–Newman–Keuls procedure [19]. These analyses were only made on the percent of the basal values.

RESULTS

This method for assessing cytosolic free calcium gave consistent values within a single day, although some variation occurred between days. The percents of basal values were consistent across days. All of the percent variances (i.e. [SEM/mean] × 100%) of these values were less than 5% within each treatment group. Thus, all of the statistical comparisons were made with the percent of basal values.

3,4-DAP altered Ca_i^{2+} under resting conditions, but not during depolarization (Figs. 1 and 2). The temporal response for 3,4-DAP was gradual com-



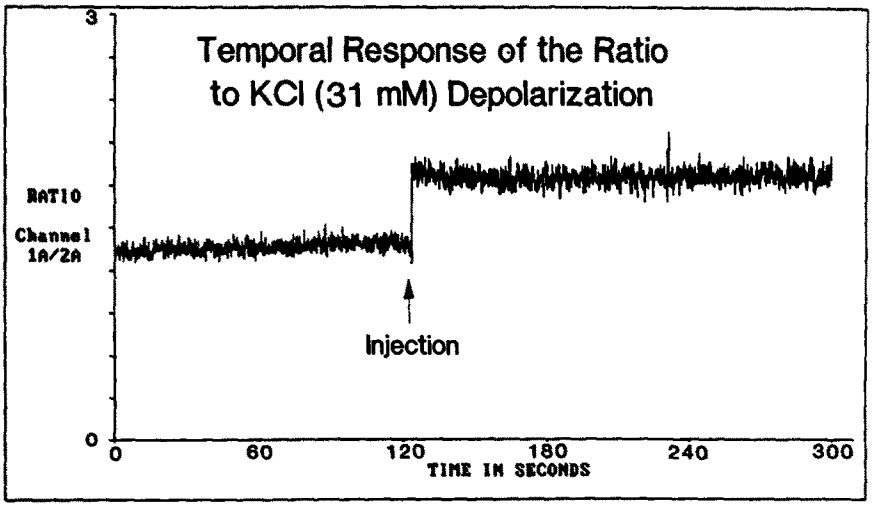


Fig. 1. Temporal response of cytosolic free calcium to 3,4-DAP and to K^+. The horizontal axis represents the time in seconds. The vertical axis represents the uncorrected ratio after excitation at 340 and 380 nm. This is a qualitative measure of Ca_i^{2+}.

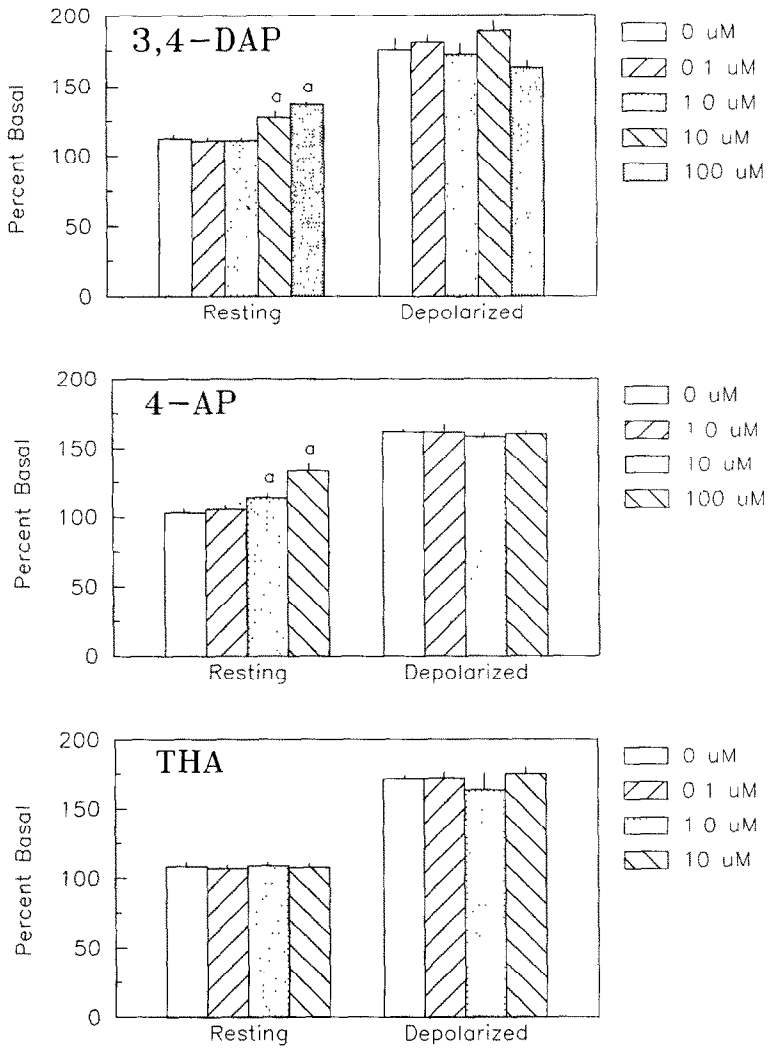


Fig. 2. Effects of 3,4-DAP, 4-AP and THA on cytosolic free calcium. Each sample was equilibrated for 3 min and then 90-sec basal values were taken. All subsequent values were divided by this number to obtain percent basal for each sample. Thus, the indicated concentration of drug was added and another 90-sec reading was taken; 31 mM KCl (final concentration) was added and samples were read for another 90 sec. Triton (final concentration 0.1%) was added, and the sample was monitored for a final 90 sec. Total time in the sample chamber was 9 min (540 sec). Basal values were 243 ± 10 (3,4-DAP, N = 24), 195 ± 4 (4-AP; N = 24) and 181 ± 8 (THA; N = 24). Each concentration of THA and 4-AP represents the mean of six observations (i.e. two experiments in triplicate). For 3,4-DAP, the number of observations were as follows: 0 μM (N = 6), 0.1 μM (N = 3), 1.0 μM (N = 6), 10 μM (N = 6) and 100 μM (N = 3). Key: (a) significantly different from all previous drug concentrations as determined by the Student–Newman–Keuls procedure.

pared to that of K^+ (Fig. 1). The mean basal value for the 3,4-DAP experiments was 243.3 ± 9.7 nM (mean ± SEM, N = 24). 3,4-DAP (0, 0.1, 1.0, 10 and 100 μM) increased Ca_i^{2+} (percent of basal) under resting conditions: 112 ± 2, 110 ± 1, 111 ± 2.5, 128 ± 3 and 137 ± 2% respectively [F = 15.0; df = 4, 19] (Fig. 2). 3,4-DAP did not alter Ca_i^{2+} after addition of 31 mM KCl: 175 ± 9, 181 ± 4, 172 ± 6, 189 ± 9 and 162 ± 4 respectively [F = 1.41; df = 4,19].

4-AP altered Ca_i^{2+} under resting conditions, but not during depolarization (Fig. 2). The mean basal value for the 4-AP experiments was 195.4 ± 3.7 nM (mean ± SEM, N = 24). 4-AP (0, 1.0, 10 and 100 μM) increased Ca_i^{2+} (percent of basal) under resting conditions: 103 ± 2.2, 105 ± 1.8, 113 ± 2.6, and 133 ± 3% respectively [F = 30.4; df = 3,20]. However, 4-AP did not alter Ca_i^{2+} (percent of basal) after addition of 31 mM KCl: 162 ± 1.6, 161 ± 4, 158 ± 1.2 and 160 ± 2.0% respectively [F = 0.37; df = 3,20].

In the concentrations examined, THA had no effect on resting Ca_i^{2+}, nor did it alter the response to K^+ (Fig. 2). The mean basal value for the THA

experiments was 180.8 ± 8.1 nM (mean ± SEM, N = 24). THA (0, 0.1, 1.0 and 10 μM) did not alter the resting Ca_i^{2+} (percent of basal): 108 ± 1.7, 107 ± 1.3, 109 ± 1.4 and 107 ± 1.8 respectively [$P > 0.05$; $F = 0.26$, df = 3,20], nor did THA alter Ca_i^{2+} during depolarization (percent of basal): 171 ± 2.8, 172 ± 3.2, 163 ± 10.3, and 175 ± 3.3% respectively [$P > 0.05$; $F = 0.69$, df = 3,20].

DISCUSSION

These results further demonstrate that the aminopyridines alter cellular calcium homeostasis. However, they do not determine whether this change is through their effects on the K^+-channel. Aminopyridines stimulate calcium uptake in non-depolarized synaptosomes [13, 20], and the present experiments demonstrate that this results in elevated cytosolic free calcium. The effects of the aminopyridines are voltage dependent [21] (i.e. they do not occur in depolarized synaptosomes), which may explain why the high K^+ values were all the same. The different temporal response between K^+ and 3,4-DAP indicates that the aminopyridines did not act instantaneously, but required more time than the K^+ depolarization. The use of K^+ to produce depolarization did not allow examination of the effects of these compounds on the return of Ca_i^{2+} to basal values (i.e. repolarization). A model which allows repolarization may reveal more about changes due to K^+ channel blockade.

The current results suggest that THA and the aminopyridines may act by different mechanisms at the tested concentrations, in spite of their structural similarities. THA [11] and 4-AP [22, 23] in high micromolar concentrations inhibit K^+ channels, whereas 3,4-DAP is about 50-fold more potent [22]. THA and 4-AP also inhibit both monoamine oxidase (MAO) and acetylcholinesterase, although 4-AP is a substantially weaker inhibitor of both enzymes. The K_i of THA for acetylcholinesterase is 1×10^{-4} M, whereas that of 4-AP is about 5×10^{-1} M. The K_i of THA for MAO is about 2×10^{-4} M [24]. By comparison, the therapeutic range of THA in serum is between 19 and 267 nM [2]. The results in this paper suggest that the beneficial effects of THA are not due to increased Ca_i^{2+}, but may be due to the more generally accepted actions of THA as a neurotransmitter degradative enzyme inhibitor. Thus, the aminopyridines may act by elevating Ca_i^{2+} and thereby increasing neurotransmitter release, in addition to weakly inhibiting the degradative enzymes.

Calcium homeostasis is altered by aging and Alzheimer's disease, and the aminopyridines partially ameliorate these deficits. Synaptosomal calcium uptake is reduced by aging and this decline is almost totally overcome by 3,4-DAP [13, 20]. The current results demonstrate that similar concentrations of 3,4-DAP elevated Ca_i^{2+}. The increase in Ca_i^{2+} may underlie the aminopyridine-induced increase in neurotransmitter release [4, 7]. Calcium uptake [25, 26], cytosolic free calcium [27] and calcium-content [28] are altered with Alzheimer's disease and the latter change is partially overcome by 3,4-DAP [14]. The current experiments demonstrate that the 3,4-DAP and 4-AP induced increase in calcium uptake elevated cytosolic free calcium. This increase in Ca_i^{2+} may underlie the beneficial clinical effects of the aminopyridines in the treatment of aging, Alzheimer's disease, and Eaton Lambert syndrome.

In conclusion, if THA and the aminopyridines both alleviate some of the symptoms of Alzheimer's disease then they may act by different mechanisms. The aminopyridines may increase Ca_i^{2+} and thus stimulate neurotransmitter release, as well as enhance other calcium-dependent processes, whereas THA may impair neurotransmitter degradation. The use of the two compounds together may be more efficacious.

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REFERENCES

1. H. Wesseling, S. Agoston, G. B. P. VanDam, J. Pasma, D. J. DeWit and H. Havinga, *New Engl J. Med.* **310**, 988 (1984).
2. W. K. Summers, L. V. Majovski, G. M. Marsh, K. Tachiki and A. Kling, *New Engl J. Med.* **315**, 1241 (1986).
3. G. E. Gibson, C. J. Pelmas and C. Peterson, *Pharmac. Biochem. Behav.* **18**, 909 (1983).
4. C. P. Peterson and G. E. Gibson, *J. Pharmac. exp. Ther.* **222**, 576 (1982).
5. H. P. Davis, A. Idowa and G. E. Gibson, *Expl Aging Res.* **9**, 211 (1983).
6. C. Eppich, C. A. Barnes and J. Baldwin, *Soc. Neurosci. Abstr.* **11**, 724 (1985).
7. C. P. Peterson and G. E. Gibson, *Neurobiol. Aging* **4**, 25 (1983).
8. J. J. McArdle, T. M. Argentieri, D. Gargano, I. Hanin and A. Fisher, *Pharmacologist* **27**, 534 (1985).
9. A. Albert, *The Acridines*, 2nd Edn. William Clowes & Sons, London (1966).
10. P. Lechat, S. Thesleff and W. C. Bowman (Eds.), *Aminopyridines and Similarly Acting Drugs: Effects on Nerves, Muscles and Synapses* (Adv. Biosci., Vol. 35). Pergamon Press, New York (1982).
11. D. R. Stevens and C. W. Cotman, *Neurosci. Lett.* **79**, 301 (1987).
12. M. I. Glavinovic, *Can. J. Physiol. Pharmac.* **65**, 747 (1987).
13. C. Peterson, D. Nicholls and G. E. Gibson, *Neurobiol. Aging* **6**, 297 (1985).
14. C. P. Peterson, R. Ratan, M. Shelanski and J. Goldman, *J. Neurochem.* **48S**, S158A (1987).
15. S. W. Leslie, J. J. Woodward and R. E. Wilcox. *Brain Res.* **325**, 99 (1985).
16. G. Gibson, T. Manger and J. Bowers, *J. Neurochem.* **48S**, SO46D (1987).
17. T. Manger, J. Bowers and G. E. Gibson, *Soc. Neurosci. Abstr.* **13**, 1238 (1987).
18. G. Grynskiewicz, M. Poenie and R. Y. Tsien, *J. biol. Chem.* **260**, 3440 (1985).
19. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics*. McGraw-Hill, New York (1960).
20. C. P. Peterson and G. E. Gibson, *J. biol. Chem.* **258**, 11482 (1983).
21. Y. Pichon, H. Meves and M. Pelhate, in *Aminopyridines and Similarly Acting Drugs: Effects on Nerves, Muscles and Synapses* (Eds. P. Lechat, S. Thesleff and W. C. Bowman), pp. 53–69. Pergamon Press,

New York (1982).
22. G. E. Kirsch and T. Narahashi, *Biophys. J.* **6**, 507 (1978).
23. Y. Matsuda, S. Yoshida, K. Fujimura and M. Nakamura, *Neurosci. Lett.* **65**, 316 (1986).
24. P. N. Kaul, *J. Pharm. Pharmac.* **14**, 243 (1962).
25. C. Peterson, G. E. Gibson and J. P. Blass, *New Engl J. Med.* **312**, 1063 (1985).
26. G. E. Gibson, P. Nielsen, K. A. Sherman and J. P. Blass, *Biol. Psychiat.* **22**, 1079 (1987).
27. C. Peterson, R. R. Ratan, M. L. Shelanski and J. E. Goldman, *Proc. natn. Acad. Sci. U.S.A.* **83**, 7999 (1986).
28. C. Peterson and J. E. Goldman, *Proc. natn. Acad. Sci. U.S.A.* **83**, 2758 (1986).