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Vitamins C and E Modulate Neuronal Potassium Currents

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Abstract. We investigated the effects of vitamins C and E on the delayed-rectifier potassium current (IK_{DR}), which is important in repolarizing the membrane potential, and on the transient A-type potassium current (IK_A), which regulates neuronal firing frequency. The whole-cell patch-clamp technique was used to measure the currents from cultured *Drosophila* neurons derived from embryonic neuroblasts. The membrane potential was stepped to different voltages between −40 and +60 mV from a holding potential of −80 mV. IK_{DR} and IK_A measured in the vitamin C-containing solution (IK_{DR} 305 ± 16 pA, IK_A 11 ± 2 pA) were smaller than those measured in the control solution (488 ± 21 pA, IK_A 28 ± 3 pA). By contrast, IK_{DR} and IK_A measured in the vitamin E-containing solution (IK_{DR} 561 ± 21 pA, IK_A 31 ± 3 pA) were greater than those measured in the control solution (422 ± 15 pA, 17 ± 2 pA). These results indicate that vitamins C and E can modulate potassium current amplitudes and possibly lead to altered neuronal excitability.

Key words: Patch-clamp — Vitamin C — Vitamin E — Potassium current

Introduction

Vitamins C and E are commonly used as antioxidants to protect against different mechanisms of cellular damage, including ischemic stroke and oxidative stress. A large number of free radicals are generated during ischemia-reperfusion, which occurs during a heart attack or a brain stroke. Ischemia prevents oxygen-rich blood from reaching cells. One consequence is increased production of free radicals, which rapidly deplete antioxidants. Ironically, during reperfusion, the rush of oxygen-rich blood to cells creates more free radicals. Vitamins C and E are used as antioxidants after cerebral ischemia and cardiac surgery. Boosting pretreatment with antioxidants attenuates ischemia-reperfusion injury (Lehr & Messmer, 1996). Intravenous injection of vitamin E 30 min prior to ischemia significantly reduces lipid peroxidation and neurological damage (Yamamoto et al., 1983), and vitamin C reduces arterial stenosius (Tomoda et al., 1996).

The antioxidant ascorbic acid, or vitamin C, does not penetrate the blood-brain barrier; its oxidized form, dehydroascorbic acid, enters the brain by means of facilitative transport. Neuronal injury in ischemic stroke is partly mediated by cytotoxic reactive oxygen species. Dehydroascorbic acid improved the outcome after stroke, and in reperfused cerebral ischemia, infarct volume was reduced in dehydroascorbic acid-treated animals (Huang et al., 2001). Ascorbic acid reversed the H_2O_2-induced inhibition of inward K current in Vicia guard cells (Zhang et al., 2001). Ascorbic acid has been reported to enhance differentiation of embryonic stem cells into neurons: it increased the expression of genes involved in neurogenesis, maturation, and neurotransmission; modulated the genes involved in cell adhesion and development (Shin et al., 2004); and caused changes of gene expression profiles during neuronal differentiation (Yu et al., 2004). The effects of ascorbic acid on membrane currents were examined in smooth muscle cells of coronary artery; application of ascorbic acid did not enhance K currents (Yamamura et al., 1999). Ascorbate (0.1 mM) did not affect inward currents, outward currents, or resting membrane potential in frog skeletal muscle fibers (Nanasi & Dely, 1995).

Vitamin E is a generic term for tocopherols (Khanna et al., 2003). Orally supplemented vitamin E reaches the cerebrospinal fluid and brain. It is essential for normal neurological function, and it is the major lipid-soluble, chain-breaking antioxidant in the body, protecting the integrity of membranes by inhibiting lipid peroxidation (Sen, et al., 2004).

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Tocopherols are predominantly found in corn, soybean, and olive oils. Oxidative stress caused by an increase in free radicals plays a role in neuronal death. Alpha-tocopherol protects neurons by reducing oxidative stress, presumably by decreasing intracellular superoxide radical (O^2) levels. It protects against cytotoxicity induced by O^{2-}, nitric oxide, and L-buthionine-[S,R]-sulfoximine, which causes depletion of intracellular glutathione (Osakada et al., 2003). Ethanol impairs neuronal culture survival, but survival was enhanced by inclusion of vitamin E in the culture medium. This elevated survival was accompanied by increased levels of anti-apoptotic proteins (Heaton et al., 2004). Alpha-tocopherol was involved in preventing glutamate-induced death of hippocampal neurons (Sen et al., 2000). Alpha-tocopherol regulates adult hippocampal neurogenesis (Cecchini et al., 2003) and decreases newborn cell death (Cuppini et al., 2001). Pretreatment with vitamins E and C prevented the reduction of sodium- and potassium-activated adenosinetriphosphatase (Na^+/K^+-ATPase) activity caused by homocysteine (Wyse et al., 2002).

The actions of vitamins C and E on voltage-dependent K channels have not been studied thoroughly, although K currents are important in controlling neuronal excitation. To fill part of this gap, the effects of vitamins C and E on the delayed-rectifier K current (IK_{DR}) and the transient A-type K current (IK_A) were investigated. Such investigation was necessary in order to establish the nature and extent of any inhibition or enhancement of the currents by vitamins C and E. Inhibition of the currents can lead to increased neuronal excitability, while enhancement can lead to decreased neuronal excitability. Modulation of the currents can have considerable implications on neural function during the use of vitamins C and E in conditions of cerebral ischemia or cardiac surgery.

Materials and Methods

Drosophila Neurons

Drosophila cultured neurons were used in this study. This neuronal preparation is well established in our laboratory and has been characterized in terms of K currents and their modulation (Alshuaib & Byerly, 1996 Alshuaib & Mathew, 1998, 2002, 2005; Alshuaib et al., 2003, 2004). Moreover, this neuronal preparation is suitable for the present experimental manipulations that include external solution changes. These cultured neurons attach strongly to the bottom of the tissue culture plate.

Preparation of Cultures

Eggs were collected over a 1.5 h period from *Drosophila melanogaster* (Oregon-R) flies maintained in pint milk bottles at 26°C. Each culture was prepared from the cells of one to three gastrulating embryos in a modified Schneider's *Drosophila* medium (DM) (Salvaterra et al., 1987). Five hours after the beginning of egg collection, the embryos were placed in a 50% ethanol/50% bleach solution for 2 min to sterilize and dechorionate them. The embryos were then repeatedly washed with DM. Two or three embryos were transferred to a drop of DM on a 35 mm tissue culture dish (Falcon 3001; Falcon, Lincoln Park, NJ). Each embryo was impaled by a hand-held micropipette (tip diameter about 100 μm); the cells were collected by suction and blown onto the surface of the dish. The cells were further dispersed by repeated passage through the tip of a smaller pipette (tip diameter 50 μm). The cells adhered to the surface of the dish within minutes of dispersal. The culture dish containing the embryonic cells (in a single drop of DM) was kept in a humid container at room temperature (23°C). All cell cultures were studied electrophysiologically 2 days (43–49 h) later at room temperature. The culture dish was used as the recording chamber with a Sylgard (Dow Corning, Midland, MI) form insert in the dish to confine the extracellular solution to a small volume (0.3 ml). Cells were viewed using Carl Zeiss (Thornwood, NY) bright-field optics.

Patch-Clamp Techniques

The conventional whole-cell patch-clamp technique (Hamill et al., 1981) was used to study the membrane currents of neurons. Electrodes were pulled from 100 μl micropipettes (VWR, Cerritos, CA), coated with Sylgard resin near the tip, and polished to a bubble number (Corey & Stevens, 1983) of 3.0–4.0. When filled with potassium aspartate solution, these electrodes had resistances of 6–12 MΩ. The application of conventional whole-cell patch-clamp to cultured embryonic *Drosophila* neurons has been described in detail previously (Alshuaib & Byerly, 1996). Typically, pipette potential was nulled, the gigaohm seal was formed using gentle suction, pipette capacitance was compensated, and the whole-cell configuration was obtained with the application of further suction.

Experiments were performed with an Axopatch 200 A-patch-clamp amplifier (Axon Instruments, Foster City, CA). Data acquisition and analysis were performed using Digidata 1200 and pCLAMP software (version 5.5, both from Axon Instruments) on a 486 Hewlett-Packard (Palo Alto, CA) personal computer. Current recordings were filtered (four-pole Bessel) at 5 kHz (capacitative currents) or 1 kHz (ionic currents) and digitized at 20 or 200 μs intervals, respectively. Passive (leakage) currents, determined from negative pulses of one-quarter the amplitude of the test pulse (-P/4), were subtracted from all of the ionic currents. The series resistance, which was the resistance of the patch electrode during the whole-cell recording, was estimated by dividing the magnitude of the 50 mV voltage step by the amplitude of the capacitive transient current. There are errors due to uncompensated series resistance of about 5 mV/100 pA.

Solutions

K^+ currents were measured in external 6K/0Ca Tris *Drosophila* saline, which contained (in mmol/l) 6 KCl, 10 $MgCl_2$, 140 Tris-HCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 glucose. The pH was adjusted to 7.4 with Tris-OH. The osmolarity was about 296 mOsm/l (measured using a 3 MO micro-osmometer; Advanced Instruments, Needham Heights, MA). The external solution was changed during experiments by pipetting 3 ml of the new solution into the 0.3 ml bath; excess solution was removed by a continuous, vacuum-powered exhaust. The new solution contained 2 mM of vitamin C and 1 mM of vitamin E. Vitamin C was dissolved in the external solution; vitamin E had to be dissolved in 150 μl of 100% ethanol before addition to the 3 ml external solution. Addition of the same amount of

Fig. 1. General properties of neurons studied. A photograph of *Drosophila* embryonic cultures, using Carl Zeiss bright-field optics. Cells had diameters of 4–7 μm.

ethanol alone to the external solution had no effect on the current. The pipette internal solution was potassium aspartate (KAsp); it contained (in mmol/l) 3 KCl, 139 L-aspartic acid, 1 $MgCl_2$, 10 HEPES, 0.1 $CaCl_2$, and 1 ethyleneglycoltetraacetic acid (EGTA, final Ca^{2+} concentration about 10 nmol/l). The internal solution was adjusted to pH 7.3 with KOH (final K^+ concentration about 156 mmol/l). The osmolarity of the internal solution was about 10% lower than that of the external solution, to improve seal formation (Hamill et al., 1981).

Statistical Analysis and Data Presentation

Population data are presented as means ± standard error of the mean (SEM). The means of two populations were compared using two-tailed Student's *t*-test for independent samples. A difference was considered statistically significant if the probability that both samples came from the same distribution was at least <0.01. Graphics were generated with Excel (Microsoft, Redmond, WA) and SigmaPlot (Jandel Scientific, Corte Madera, CA) software packages.

Results

Neurons were identified by the presence of one or more thin processes. Neurons with clearly visible cell bodies, usually alone, were selected for study. The typical cell studied had one process with a cell body 4–7 μm in diameter. Occasionally, cells with two or three processes were studied. Examples of the types of cells studied are shown in Figure 1. Cells were studied between 43 and 49 h from the time of egg laying (23°C).

Aspartate was found to be the best anion to use in the intracellular solution during whole-cell experiments with potassium aspartate internal solution. We calculated the total capacitance for each cell by integrating the capacitive current flowing in response to a 50 mV hyperpolarizing step. The cell capacitance was 8.12 ± 1.61 pF ($n = 17$); it gives a measure of cell membrane area. The resting membrane potential was 77.3 ± 0.5 mV ($n = 17$). Whole-cell resistance (R_{in}) was measured by stepping the membrane potential from −60 to −110 mV and dividing this 50 mV step by the measured current amplitude between 90 and 100 ms. R_{in} was 8.19 ± 0.91 GΩ ($n = 17$).

IK_{DR} Properties

Potassium current was measured in a Ca^{2+}-free *Drosophila* external solution because IK_{DR} is smaller in Ca^{2+}-containing solutions than in Ca^{2+}-free solutions due to contamination of the outward IK_{DR} by the inward Ca^{2+} current (Alshuaib & Mathew, 1998). Moreover, neuronal K^+ current in *Drosophila* does not have a Ca^{2+}-dependent component (Alshuaib & Byerly, 1996).

Figure 2 shows typical examples of IK_{DR} recorded from neurons at potentials from −40 to +60 mV. IK_{DR} was calculated between 490 and 500 ms (steady state) of the pulse to exclude any possibility of A-current contribution to the measured amplitude. All neurons displayed the delayed-rectifier (noninactivating) K^+ current. We applied a voltage protocol that maximizes the delayed-rectifier K^+ current and diminishes the A-type (inactivating) K^+ current (holding potential −80 mV, test pulses −40 to +60 mV) (Byerly & Leung, 1988; Alshuaib & Mathew, 1998). IK_{DR} was clearly activated at 0 mV but only weakly activated at −20 mV. IK_{DR} typically reached its peak within 10–25 ms after the start of the pulse. The time course of inactivation was quantified by calculating the percentage of the peak current that had inactivated at 500 ms.

Effects of Vitamins C and E on IK_{DR}

The main purpose of this study was to investigate the effects of vitamins C and E on the K^+ current. These effects occurred immediately after vitamin addition to the external solution; they were observed as soon as we measured the current (within 10 s) and remained the same for the duration of the recording (about 10 min). IK_{DR} measured in the vitamin C-containing solution (305 ± 16 pA, $n = 20$) was smaller than that measured in the control 6K/0Ca Tris solution (488 ± 21 pA, $n = 20$) ($P < 0.0001$) (Fig. 2). By contrast, IK_{DR} measured in the vitamin E-containing solution (561 ± 21 pA, $n = 18$) was greater than that measured in the control 6K/0Ca Tris solution (422 ± 15 pA, n = 18) ($P = 0.0001$) (Fig. 2). IK_{DR} steady-state inactivation was not affected by vitamin C (13.5 ± 1%, $n = 14$) compared to control solution (12.8 ± 1%, $n = 14$); inactivation was increased by vitamin E solution (36.4 ± 2%, $n = 14$) compared to control solution (9.2 ± 1%, $n = 14$; $P < 0.0001$; 60 mV pulse). Figure 3 shows the current-voltage (I-V) relations of IK_{DR} measured in control 6K/0Ca Tris solution, vitamin C-containing 6K/0Ca Tris

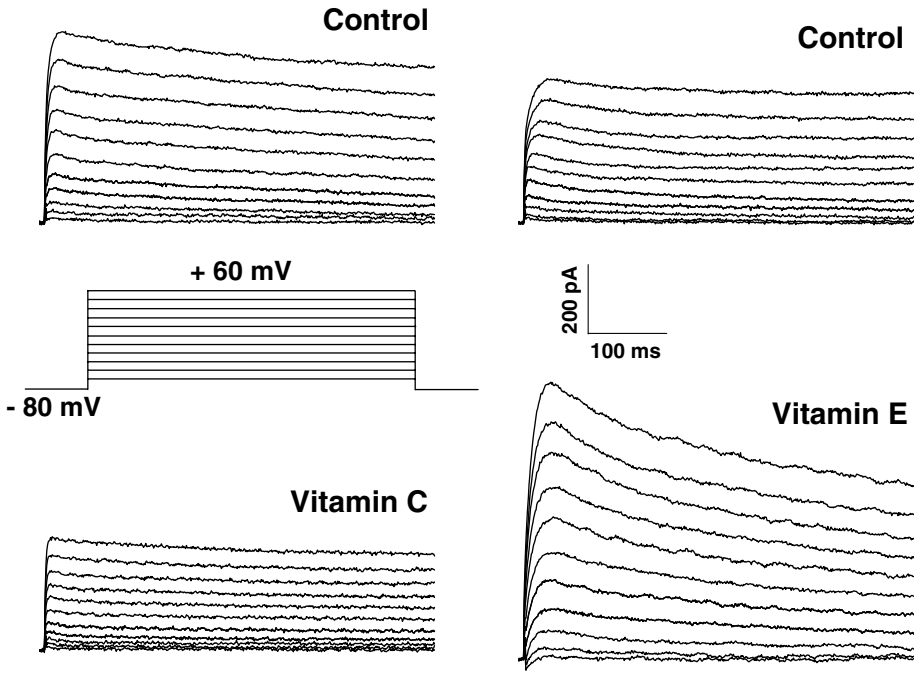

Fig. 2. Effect of vitamins C and E on IK_{DR} of *Drosophila* neurons in control 6K/0Ca Tris *Drosophila* saline and in vitamin-containing (either C, 2 mM, or E, 1 mM) *Drosophila* saline. K^+ currents were recorded from neurons during 11 clamp potentials of 500 ms between −40 mV (*bottom trace*) and +60 mV (*top trace*) in steps of 10 mV. Holding potential was −80 mV. Current traces are from the same neuron before and after vitamin application. The pipette contained potassium aspartate solution.

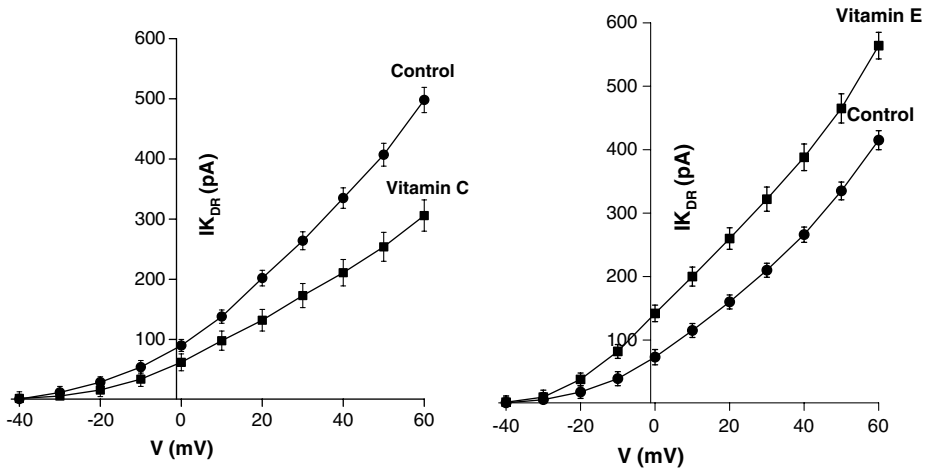

Fig. 3. Current-voltage (I-V) relations of *Drosophila* neuronal IK_{DR} in control 6K/0Ca Tris *Drosophila* saline and in vitamin-containing (either C, 2 mM, or E, 1 mM) *Drosophila* saline. The pipette contained potassium aspartate solution. Current values are from the same neurons before and after vitamin application. Data points are mean ± SEM. The mean IK_{DR} was determined at steady state (490–500 ms of pulse).

solution, and vitamin E-containing 6K/0Ca Tris solution. Due to the difficulty of keeping a whole-cell patch-clamp recording during external solution changes, reversibility of vitamin effects on the current was not tested.

IK_A Properties

To activate the A-type current, a 1 s prepulse of −110 mV was necessary before the +20 mV (500 ms) test pulse. Such preconditioning hyperpolarization removes inactivation of IK_A channels and maximally elicits IK_A (Alshuaib et al., 2001). Figure 4 illustrates the method used to isolate IK_A. The A current was not elicited when the prepulse (1 s) was −30 mV. Rather, a prepulse of −110 mV was necessary to elicit IK_A.

Effects of Vitamins C and E on IK_A

IK_A measured in the vitamin C-containing solution (11 ± 2 pA, $n = 19$) was smaller than that measured in the control 6K/0Ca Tris solution (28 ± 3 pA, $n = 19$) ($P = 0.0001$) (Fig. 4). IK_A measured in the vitamin E-containing solution (31 ± 3 pA, $n = 14$) was greater than that measured in the control 6K/0Ca Tris solution (17 ± 2 pA, $n = 14$) ($P = 0.0006$) (Fig. 4). Activation time of IK_A was similar in control 6K/0Ca solution (12.4 ± 1 ms, $n = 14$), vitamin C-containing solution (13.6 ± 1 ms, $n = 14$), and vitamin E-containing solution (12.9 ± 1 ms, $n = 14$). Thus, IK_A amplitude was calculated between 10 and 20 ms of the pulse. Inactivation of IK_A was considered when the half-decay time was faster in vitamin C-containing solution (20 ± 2 ms,

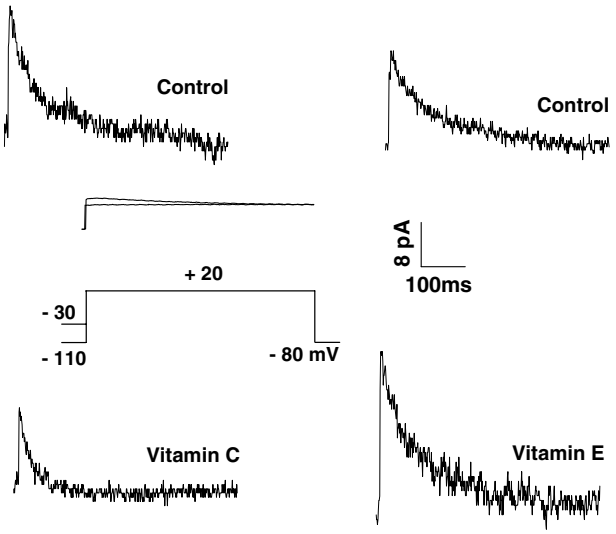

Fig. 4. Effect of vitamins C and E on IK_A in *Drosophila* neurons. The current was measured before and after vitamin C (2 mM) or vitamin E (1 mM) application. IK_A was obtained by subtracting the sustained current from the total current. *Middle part:* The *upper trace* (total current) was elicited by a 500 ms step to +20 mV from a 1 s prepulse of −110 mV. The *lower trace* (sustained current) was elicited by a 500 ms step to +20 mV from a 1 s prepulse of −30 mV, where IK_A is inactivated. Pipette solution was potassium aspartate and external solution was either control 6K/0Ca *Drosophila* Tris saline or vitamin-containing 6K/0Ca *Drosophila* Tris saline.

$n = 14$) than in control solution (40 ± 3 ms, $n = 14$) ($P < 0.0001$). Half-decay time was similar in the vitamin E-containing (54 ± 5 ms, $n = 14$) and control (57 ± 4 ms, $n = 14$) solutions.

Discussion

The relatively unknown effects of vitamins C and E on membrane currents led us to investigate the effects of these vitamins on K current in an effort to understand K^+ channel modulation that may consequently mediate alterations in neuronal function. Although the study was performed on *Drosophila* neurons, the basic IK_{DR} and IK_A properties are similar to those of mammalian neurons (Alshuaib & Mathew, 1998; 2004; Alshuaib et al., 2001). Vitamin C has been shown previously to reverse the H_2O_2-induced inhibition of inward K current in Vicia guard cells (Zhang et al., 2001). Previous studies indicated that the calcium and sodium inward currents were not altered by either vitamin C (Nanasi & Dely, 1995; Parsey & Matteson, 1993) or vitamin E (Collins & Hilgemann, 1993). The present study showed that vitamin C reduces the transient and sustained K currents, which could increase neuronal excitability, and that vitamin E increases the transient and sustained K currents, which could inhibit neuronal excitability.

Mechanism of IK_{DR} and IK_A Inhibition by Vitamins C and E

There is little published work on the effects of vitamins C and E on the gating of K^+ channels. Vitamin binding at a specific site and obstructing the pore when present in the channel represents interactions with channel gating mechanisms. The alterations of IK_{DR} and IK_A could be caused by direct effects of vitamins C and E on K^+ channels. The open probability of these channels could be altered or the single-channel current amplitude could be altered. Single-channel recording can be used to determine how the channel is affected for each vitamin. To determine whether any vitamin interacts directly with the channel protein, the vitamin should be applied to excised patches to test if it can alter channel activity without mediation of second messengers.

Modified IK_{DR} and IK_A Can Alter Neuronal Function

Our study shows that vitamin C can increase and vitamin E can decrease neuronal excitability in *Drosophila* neurons. These modifications should be considered in the context of neuronal function. The sustained IK_{DR} current is responsible for repolarization during the falling phase of the action potential. With a smaller IK_{DR}, the action potential is prolonged. For example, a decreased IK_{DR} in the soma can enhance neurotransmitter release at the nerve terminal. IK_A regulates the membrane potential between action potentials (neuronal firing threshold) and thus affects the rate of repetitive action potentials. With a smaller IK_A the refractory period is expected to be shorter and the firing frequency to be enhanced. Collectively, the inhibition of IK_{DR} and IK_A by vitamin C can increase calcium influx via voltage-gated calcium channels into the neuron, and this can have a wide array of functional consequences. Since this is the first report of vitamin C inhibition of K currents, it becomes necessary to examine the effects of oral vitamin C administration in mammals on neuronal K currents and intracellular Ca^{2+} concentration. This will reveal the involvement of vitamin C in regulating intracellular Ca^{2+} level.

The enhancement of IK_{DR} and IK_A by vitamin E can reduce the periods during which the neuron is depolarized. In this regard, it would seem that vitamin E plays a neuroprotective role since its enhancement of IK_{DR} and IK_A can possibly prevent intracellular Ca^{2+} overload. Increased intracellular Ca^{2+} level has been reported in aging motor nerve terminals (Alshuaib & Fahim 1990) and may be implicated in neurodegenerative diseases.

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