Age, Vol. 13, 65-71, 1990

NIFEDIPINE INFLUENCES ROTIFER LIFESPAN: STUDIES ON THE CALCIUM THEORY OF AGING

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ABSTRACT

The results presented here show that there is an increase in calcium uptake in the cells of the rotifer *Asplanchna brightwelli* as it ages. The results further show that the 1,4-dihydropyridine calcium channel blocker nifedipine prevents this age-related increase in calcium uptake when administered to rotifers at a concentration of 1.0 μM. A range of nifedipine concentrations from 0.1 μM to 5.0 μM significantly increased rotifer lifespan. Thus, prevention of an age-associated increase in calcium uptake is correlated with increased lifespan in rotifers. These findings suggest that there is an age-related decline in calcium homeostasis, and support the theory that calcium ion regulation is a vital factor in aging rotifer tissues.

INTRODUCTION

This study was designed to examine age-related patterns of calcium uptake in the rotifer *Asplanchna brightwelli* and to determine whether calcium uptake or lifespan of the rotifer would be influenced by treatment with the calcium channel blocker nifedipine.

There is mounting evidence that cellular and physiological changes in aging are linked to altered calcium homeostasis (1). The present study was undertaken to explore the relationship between calcium and aging, and in particular to test the theory that altered calcium homeostasis could influence lifespan in an invertebrate system, the rotifer.

Calcium is a basic regulator of intracellular function in all organisms. As such, it is responsible for the control of a large number of biochemical processes within cells (2, 3). In healthy cells a number of mechanisms exist to maintain calcium homeostasis. Excess calcium entering via calcium channels can be pumped out by membrane calcium-ATPase proteins (2, 3). Alternatively, excess calcium can be sequestered by calmodulin or in the mitochondria and endoplasmic reticulum (2, 3, 4).

In aging, necrotic and ischemic cells, the ability to maintain calcium homeostasis declines (5-7), producing either an excess or a deficiency of intracellular calcium. When the cell's ability to sequester influxing calcium decreases, this leads to an increase in intracellular calcium concentration (1, 8, 9). The elevation of intracellular calcium associated with aging in some cell types appears to be toxic. Seisjo (6) found that if the intracellular calcium concentration remains high for prolonged periods, cell death results. Increased intracellular calcium also appears to be an important factor in the initiation of cell death following cell injury (10). In marked contrast, endocrine cells of aged vertebrates appear to lack sufficient intracellular calcium and to display impaired hormone mobilization (11). In these endocrine cells, function can be restored to presenescence levels if the calcium concentration within the cell is raised (12).

Nifedipine is a well-known calcium channel blocker (13). A potential method of reducing calcium fluxes and of artificially maintaining calcium homeostasis would be to limit the entry of calcium through calcium channels in the cell membrane. Administration of dihydropyridine calcium channel blockers to neurons immediately prior to and during cell injury has been found to improve cell function, probably by delaying the cytotoxic events initiated by excess intracellular calcium accumulation (14, 15). If intracellular calcium buildup is associated with aging, limiting calcium entry through channels could delay cell aging and potentially increase lifespan.

Massie *et al.* (16) report that total body calcium levels increased by 115% during the adult lifespan of *Drosophila*. They found that the lifespan of the *Drosophila* decreased as dietary calcium was increased. Massie *et al.* (16) attempted to reduce the age-related increase in calcium by administering the calcium channel blocker nifedipine. They report that administration of 1 mg/ml nifedipine starting at 2 weeks produces a small but significant (5.4%) increase in *Drosophila* lifespan.

Experiments conducted by Sincock (17, 18) and Lansing (19) suggested that rotifers tend to accumulate calcium as they age. The purpose of this study was to establish whether changes in calcium uptake occur with age in the rotifer; it was also to determine whether the administration of the calcium channel blocker nifedipine could alter calcium ion transport in the rotifer, or alter rotifer lifespan. The effect of nifedipine on rotifer reproduction, activity level and phototaxis was also examined.

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RESULTS

The initial experiments were performed on groups of 24 rotifers to establish the normal lifespan of *Asplanchna brightwelli;* it was 5.10 + 0.25 days. *A. brightwelli* was then exposed to a range of concentrations of the calcium channel blocker nifedipine in order to determine the drug's effect on rotifer lifespan. Table 1 shows the data from experiments in which a range of nifedipine concentrations from 0.1 μM to 10 μM were tested. A one-way analysis of variance followed by a post-hoc Tukey test showed that concentrations of nifedipine between 0.1 and 5 μM produced a significant increase in rotifer lifespan compared to the untreated controls ($F(5, 138) = 13.909$, $p<0.001$). Higher doses of the drug had either no effect on lifespan or significantly shortened lifespan. Similar results were obtained in all three series of replicate experiments.

Survivorship curves for the control and for two experimental groups are displayed in Fig. 1. The curves for rotifers treated with 0.5 μM and 1.0 μM doses of nifedipine show a clear shift to the right of the control group indicating their longer lifespan. The data in Table 1 show that rotifer lifespan was enhanced over a range of concentrations (0.1 μM — 5.0 μM). Since there was no significant difference between these four groups, a concentration of 1.0 μM nifedipine was selected for all subsequent experiments.

The data in Table 1 indicates that the increase in lifespan observed in the rotifers was primarily due to an increase in the length of the prereproductive period. This increase was found to be significant compared to the untreated controls ($F(5, 138) = 15.872$, $p<0.001$). The rotifer lifespan consists of the prereproductive, reproductive and post-reproductive period. Nifedipine had no significant effect on the length of the reproductive period or post-reproductive period, or on the number of offspring produced by the rotifers (data not included).

The nifedipine used in all experiments was dissolved in 95% ethanol. Since ethanol is known to have free radical scavenging capability, control experiments were carried out to compare the mean lifespan of rotifers treated with ethanol with that of control animals. A one-way analysis of variance followed by a post-hoc Tukey test showed that there was no significant difference between the groups ($F(2, 69) = 0.201$, $p>0.5$). At the concentrations used here, ethanol does not affect rotifer lifespan.

Influence of Nifedipine on Activity

The experiments in this section were designed to determine the activity level of control and nifedipine-treated rotifers. The experimental animals were exposed to 1.0 μM nifedipine. In the first experiment, the number of grids that control and experimental rotifers traversed in a one-minute time period was counted. Table 2 displays the mean movement count on each day throughout the lifespan of the organism; it can be seen that movement declines with age. One-way analysis of variance showed that for control rotifers activity declines significantly late in life ($F(5, 24) = 3.959$, $p<0.05$). One-way analysis of variance showed that the nifedipine-treated rotifers also showed a decrease in movement with increasing age ($F(6, 28) = 10.142$, $p<0.001$). Fig. 2 illustrates the general decrease in rotifer activity with age in both groups, and illustrates that the rotifers treated with nifedipine showed reduced activity as compared to controls.

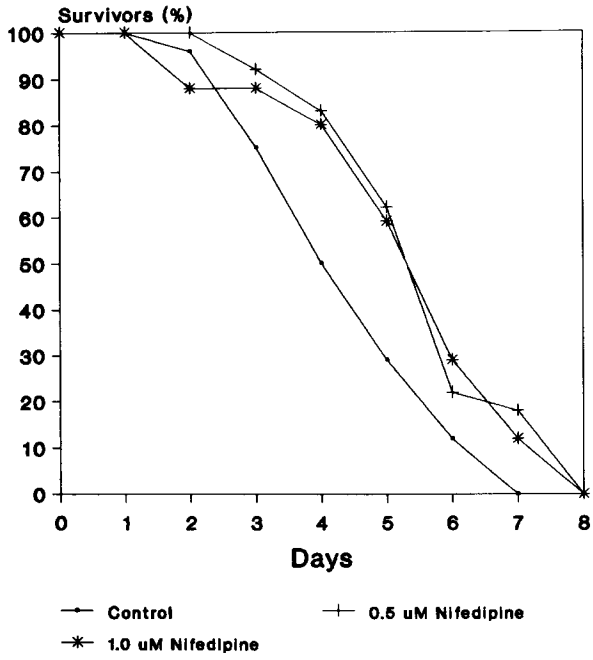

Fig. 1: Survivorship curves of rotifers exposed to different concentrations of nifedipine compared to untreated control. (n = 24)

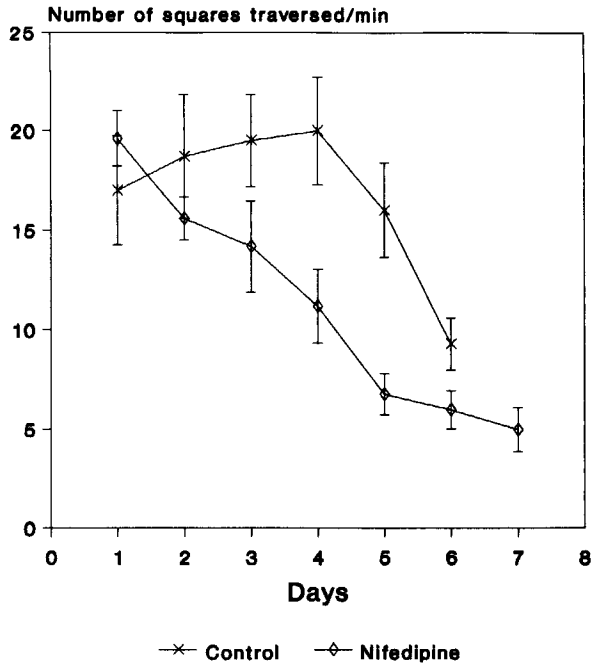

Fig. 2: Movement counts of *A. brightwelli* exposed to different concentrations of nifedipine. (Movement expressed in mean number of squares transversed per minute.) (n = 24)

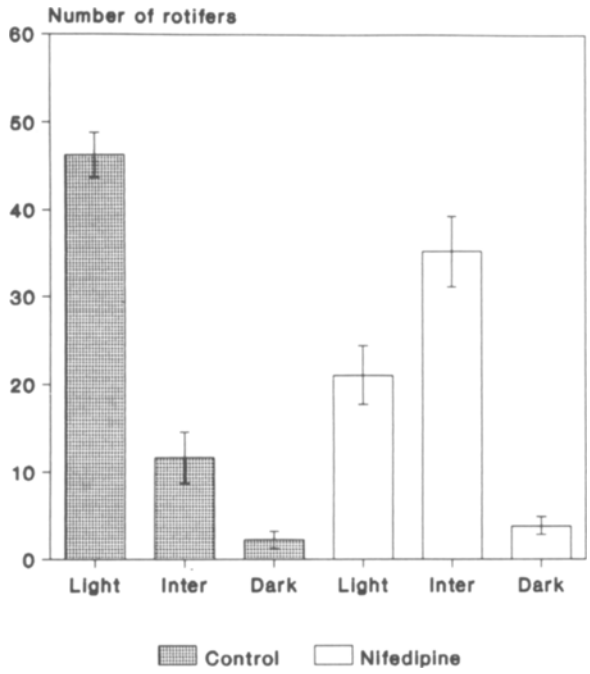

Fig. 3: Bar graph comparing the movement of control and nifedipine-treated *A. brightwelli* from the intermediate chamber of a three-chambered box lit from one end. (n = 5) Nifedipine concentration = 1 micromolar

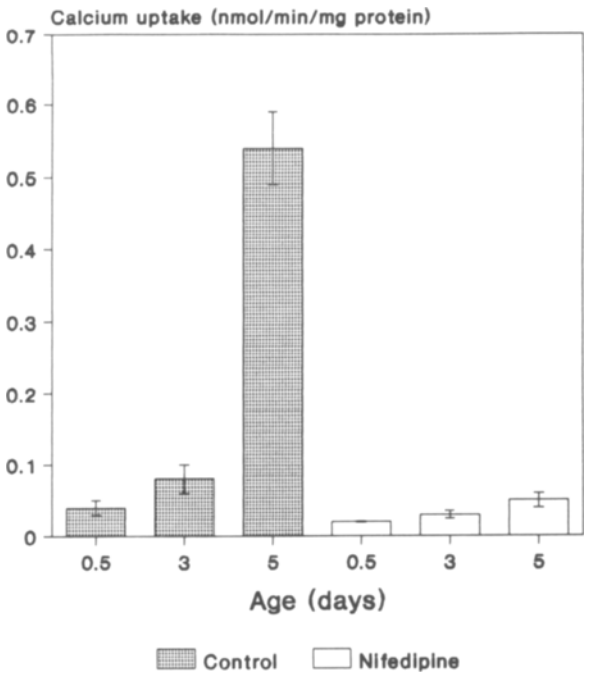

Fig. 4: Bar graph comparing the uptake of calcium ion by the dispersed cells of control and nifedipine-treated rotifers of three different age groups. (Mean uptake expressed in nanomoles of calcium per minute per milligram of rotifer protein.) (n = 3) Nifedipine concentration = 1 micromolar

3-day-old rotifers were placed in the central chamber of a three-chambered rectangular dish and were left to migrate towards a beam of light placed at one end of the dish. The experiment was repeated using rotifers exposed to nifedipine since birth.

The second experiment to measure activity made use of the positive phototactic response of rotifers. Sixty 3-day-old rotifers were placed in the central chamber of a three-chambered rectangular dish and were left to migrate towards a beam of light placed at one end of the dish. The experiment was repeated using rotifers exposed to nifedipine since birth.

Fig. 3 compares the mean number of control and treated rotifers found in each chamber following a 10-minute exposure to the light. A one-way analysis of variance showed that significantly fewer nifedipine-treated rotifers moved towards the light source ($F(2, 12) = 20.557$, $p<0.01$). The bar graph in Fig. 3 shows that most of the nifedipine-treated rotifers remained in the central chamber where they were initially placed.

The results of these two experiments indicate that nifedipine significantly reduces both the activity level and the phototactic response of rotifers exposed to the drug throughout their lifespan.

Uptake of ^{45}Ca by Rotifer Cell Suspensions

In this series of experiments, the effect of nifedipine on the uptake of ^{45}Ca by dispersed rotifer cells of three different age groups was examined. The rotifer protein was first measured using a standard Bradford's assay, so that the data could be expressed in terms of calcium uptake per mg of rotifer protein.

The mean calcium ion uptake in one minute by one milligram of rotifer protein is shown in Fig. 4. A one-way analysis of variance test performed on this data, followed by a post-hoc Dunnett's test showed that there was a significant increase in the rate of calcium uptake in control rotifers with increasing age ($F(2,5) = 64.334$, $p<0.01$). This same test performed on the nifedipine treated rotifers showed no significant increase in calcium ion uptake with age ($F(2, 5) = 2.58$, $p>0.05$). A two-way analysis of variance revealed that the combined effect of age and nifedipine treatment was highly significant: at 0.5 days the difference in calcium uptake by control and experimental animals is very small, while the difference in the 5-day-old rotifers is large ($F\ 2,10 = 14.9$, $P<0.001$).

DISCUSSION

The results presented here show that exposure of rotifers to low concentrations of the calcium channel blocker nifedipine can significantly extend their lifespan, by as much as 24%. There are several possible explanations for the action of nifedipine on lifespan. One explanation could be that nifedipine slows development by non-specific toxicity; this could account for the slowing of the prereproductive or growth period of the rotifers observed here. If this were so, increased doses of nifedipine should show increased toxicity. In fact, no toxic effect was observed until the nifedipine concentration reached the very high level of 0.01 M. Moreover, nifedipine did not alter reproduction, as would be expected of a toxic chemical. It appears that a toxic, hormeotic effect cannot explain these results.

Another possible explanation of nifedipine action could be that it reduces rotifer activity, thus conserving energy for life maintenance, in line with the rate of living theory of aging. The results presented here show that rotifers treated with nifedipine do move more slowly than control rotifers. A similar lifespan extension was observed when rotifer activity was reduced by low dosages of curare (20). The reduced activity observed as a result of nifedipine treatment may indicate that neurotransmitter release is affected by the calcium channel blockade. Miller (21) found that the application of calcium channel blockers to rats resulted in a decrease in acetylcholine release and in muscle relaxation. Since the presence of acetylcholine in rotifers has been established (22) it is possible that nifedipine treatment may interfere with acetylcholine release, thus slowing movement. The finding that nifedipine decreases the rotifers' ability to move toward light also suggests that nifedipine acts on the neurons or muscles of the rotifer. Positive phototaxis in rotifers has been described by Cornillac *et al.* (23), while the neuromuscular pathway involved has been described by Clement *et al.* (24). Calcium channel blockade could reduce either neurotransmission or muscle contraction to produce the slowing of activity observed in this study. However, it is difficult to explain the lifespan extension observed here in relation to the rate of living theory of aging when other studies show that a simple rate of living relationship does not apply to rotifers (25) and that the rate of living theory has been questioned in its broader applications (26).

The most probable explanation of the nifedipine-induced lifespan extension is that this drug is acting on the rotifers by its known biological specificity as a calcium channel blocker. In this context the results presented here could be interpreted in relation to the calcium theory of aging.

The idea that calcium is implicated in rotifer aging was first advanced by Lansing (19) and further examined by Sincock (17, 18). The results presented here show that calcium uptake is greatly increased in normal, untreated rotifers at the end of their lifespan. It appears from these results that the ability to exclude calcium is greatly reduced by the aging process. An increased calcium concentration is characteristic not only of aging cells, but also of necrotic and ischemic cells (5-7). Khachaturian (27) notes that Seisjo's (6) work on ischemia provides an important link between intracellular calcium accumulation and the free radical theory of aging.

Seisjo (6) suggests that ischemia, like aging, results in a buildup of toxic free radicals that cause lipid peroxidation of plasma membranes. Similar findings have been reported by Farber (5) and Trump *et al.* (10). The resultant membrane damage may cause a disruption in the permeability barrier to calcium ions (5). Seisjo (6) further notes that the activation of phospholipase enzymes by calcium may cause further membrane breakdown, the generation of more free radicals, and ultimately, cell death. Khachaturian (28) has developed a "calcium hypothesis of brain aging." His hypothesis that sustained changes in calcium homeostasis provide a final common pathway for age-associated brain changes could pertain to other tissues as well.

It seems likely that lipid peroxidation of aging rotifer membranes is an important factor in the calcium ion accumulation reported here. Sawada and Carlson (29) found that there is a dramatic increase in lipid peroxidation of the membranes of *A. brightwelli* as the rotifer ages. The attendant membrane damage and calcium influx may be an important trigger for the initiation of cell death. In this context, it is interesting to note that nifedipine reduces lipid peroxidation levels in microsomes (30) and in liposomes (31). Engineer and Sridhar (30) report that microsomal lipid peroxidation in rat heart and liver, measured by malondialdehyde formation, was inhibited by nifedipine over a wide range of concentrations (47 μM to 6 mM). They suggest that nifedipine is diverting electrons away from the path leading to lipid peroxidation. Ondrias *et al.*(31) report that nifedipine is an effective free radical scavenger, only 10 times less effective than BHT. If

Table 1. The effect of nifedipine on the lifespan and prereproductive period of *A. brightwelli.* (n=24)

Nifedipine concentration	Prereproductive period	Mean lifespan (days ± S.E.M.)
0	2.5 ± 0.1	5.1 ± 0.3
0.1 μM	3.4 ± 0.3[a]	6.3 ± 0.1[a]
0.5 μM	3.3 ± 0.3[a]	6.3 ± 0.1[a]
1.0 μM	3.2 ± 0.2[a]	6.1 ± 0.1[a]
5.0 μM	3.0 ± 0.2[a]	6.0 ± 0.2[a]
10.0 μM	2.5 ± 0.4	5.3 ± 0.2

[a]Significantly different from control
Prereproductive period: $F(5, 138) = 15.872$, $p<0.001$
Lifespan: $F(5, 138) = 13.909$, $p<0.001$

Table 2. The effect of nifedipine on the activity level of *A. brightwelli* (expressed as the number of grids transversed in a one minute period) (n=5)

Age (days)	# Grids ± S.E.M. Control	1.0 μM Nifedipine
1	17.0 ± 2.7	19.6 ± 1.4
2	18.7 ± 3.1	15.6 ± 1.1
3	19.5 ± 2.3	14.2 ± 2.3[a]
4	20.0 ± 2.7	11.2 ± 1.9[a]
5	16.0 ± 2.4	6.8 ± 1.0[a]
6	9.3 ± 1.3[a]	6.0 ± 1.0[a]
7	—	5.0 ± 1.1[a]

[a]Significantly different from day 1 count
Control: $F(5,24) = 3.959$, $p<0.05$
Nifedipine: $F(6, 28) = 10.142$, $p<0.001$

nifedipine is acting as a free radical scavenger, our results could also be interpreted in relation to the free radical theory of aging (32). Vitamin E and other free radical scavengers extend lifespan in rotifers (33-35). Since the concentrations of nifedipine used in these *in vitro* studies were higher than in our studies, further experimentation would be required to examine this radical-scavenging effect.

The results show that administration of nifedipine stopped the age-related influx of calcium into rotifer cells and concomitantly increased the lifespan of the organism. These results are in agreement with those of Massie *et al.* (16) who show a similar effect of nifedipine in increasing *Drosophila* lifespan. The findings reported here are in accordance with the idea that altered calcium homeostasis and altered calcium sequestration or mobilization are important factors in the aging process, and may be modifiable by appropriate drug treatment.

EXPERIMENTAL PROCEDURES

The short lived rotifer *Asplanchna brightwelli* clone B461, originally obtained from Dr. J. Gilbert, Dartmouth College, Hanover, New Hampshire, was the organism used in this study. The rotifers were fed *Paramecium caudatum* cultured in a cerophyll infusion medium and incubated with *E. coli* as described previously (33).

To begin any experiment, each well of a 24-well tissue culture dish (No. 76-063-05; Flow Laboratories, McLean, Virginia) was filled with 2.5 ml of fresh medium. Adult rotifers were collected and placed singly into the wells where they reproduced parthenogenetically. The newborn rotifers (0-1 hr old), whose time of birth was precisely known, were harvested, transferred into new individual culture chambers, and divided into the various experimental groups.

Lifespan Experiments

Nifedipine (Lot N-763; Sigma Chemical Company) was dissolved in 95% ethanol. Medium was prepared by dissolving nifedipine stock solution into fresh medium to obtain concentrations ranging from 0.1 μM to 10 μM.

Newborn rotifers were transferred into each of the 24 wells of new culture dishes containing either medium with nifedipine or control medium. The culture dishes were incubated at 19°C and the chambers were checked every six hours for deaths and for the presence of offspring, which were counted and discarded. Additionally, the medium in each well was replaced with freshly prepared medium, to ensure a constant food supply, and to circumvent the breakdown of the light sensitive nifedipine (36).

Determination of Rotifer Activity

The activity level of rotifers in both control and experimental groups was determined in two ways: by counting movement across the squares of a grid, and by counting the number of organisms moving towards light in a fixed period of time.

In the first set of experiments, 24 control and 24 nifedipine-treated rotifers were placed as usual in individual wells of a tissue culture dish. The dish was placed on a transparent plastic sheet upon which was drawn a 0.5 mm grid network. The rotifers were observed under a dissecting microscope, and the number of grids they traversed in a 1-minute time period was recorded (20).

In the second set of experiments, movement of the rotifers depended upon their positive phototactic response (24). Sixty rotifers were placed in the middle chamber of a rectangular three-chambered box with dimensions 12 cm x 5 cm x 3 cm. All sides of the box were blackened except for a small slit at one end, at which a beam of light was placed. The animals were dark-adapted for 10 minutes, after which the light was turned on and the partitions between the chambers removed. The rotifers were left for a further 10 minutes; the partitions were then replaced, and the rotifers in each chamber counted (23).

Uptake of ^{45}Ca by Rotifer Cell Suspensions

Two groups of rotifers were used for this set of experiments. The control group was raised in normal medium while the experimental group was raised in medium containing 1.0 μM nifedipine. During all experimental procedures, the control and experimental rotifers were kept in phosphate buffer and phosphate buffer with 1.0 μM nifedipine respectively.

Control and experimental rotifers were cultured and separated into three age groups: 0.5 day, 3 days and 5 days old. The 0.5-day-old samples each comprised 100 rotifers while three- and five-day-old samples contained 50 rotifers. Each experiment in the following section was repeated twice.

To obtain cell suspensions, rotifers in each sample were homogenized in 100 μl of phosphate buffer or phosphate buffer with nifedipine. Following homogenization 300 μl of 0.25% trypsin in normal saline (Gibco Laboratories, Grand Island, New York) was added and the samples were placed in a shaker bath at room temperature for 30 minutes. The samples were then centrifuged for 15 minutes at 1500 g and the pellets resuspended in 100 μl of the appropriate phosphate buffer. Visual microscopic examination showed that whole cells were present, not cell fragments. A Bradford's protein assay (37) was run on 20 μl of each whole cell suspension.

After the protein concentrations in each sample had been established, the amount of cell suspension corresponding to 200 μg of rotifer protein was taken from each sample and placed in separate tubes. The volume of each was then made up to 60 μl with appropriate amounts of buffer. The samples were left to incubate with 103 μl of $^{45}CaCl_2$ for 20 minutes. This was equivalent to 0.1 μCi or 100,000 cpm. The $^{45}CaCl_2$ was obtained from Amersham (Oakville, Ontario) at a concentration of 1 mCi/ml and specific activity of 20 mCi/mg Ca.

After incubation, each sample of the cell suspensions was separately placed in a sterile syringe and filtered through an 8 μM millipore SC type filter (Millipore

Corporation, Bedford, MA). The samples were then washed twice with 10 ml of either phosphate or phosphate–nifedipine buffer to ensure the removal of any ^{45}Ca label not taken up by the cells. The filters were then placed in liquid scintillation vials with 2.0 ml of scintillation fluid, vortexed for 30 seconds, and counted for radioactivity in a liquid scintillation counter (Nuclear Chicago, Mark III). The methodology has been more extensively described Sawada (38).

Statistical Analysis

All lifespan data was analyzed using a one-way analysis of variance followed by a post-hoc Tukey test (39). Other data was analyzed using a post-hoc Dunnett's test and two-way analysis of variance (39).

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