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Effects of Nano Red Elemental Selenium on Sodium Currents in Rat Dorsal Root Ganglion Neurons

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Abstract—**Nano red elemental selenium(Nano-Se), was demonstrated to be useful in medical and scientific researches. Here, we investigated the effects of Nano-Se on sodium currents on rat dorsal root ganglion neurons (DRG), using the whole-cell patch clamp method. Nano-Se reversibly decrease the $I_{Na(TTX-S)}$ in a concentration-dependent, time-dependent and open–channel block manners without affecting $I_{Na\ (TTX-R)}$. It shifted the steady-state activation and inactivation curves for I_{Na} to more negative potentials. In the research of recovery from inactivation, the recovery time constant is longer in the present of Nano-Se. Nano-Se had a weaker inhibitory effect on $I_{Na,}$ compared with marked decrease caused by selenite which indicated that Nano-Se is less neurotoxic than selenite in short-term/large dose treatments and had similar bioavailability to sodium selenite. The results of interaction between the effects of Nano-Se and selenite on sodium currents indicated a negative allosteric interaction between the selenite binding site and the Nano-Se binding site or that they have the same competitive binding site.**

I. Introduction

Selenium, as one of the essential elements for the health of mammalian animals, has key functions in the balancing of the redox system, the proper function of immune system, and anticarcinogenetic effects. Selenium has several forms, such as sodium selenite(Na_2SeO_3), ebselen, and other selenium-containing organic compounds. However, a few studies have been focused on Se in zero redox state (Se^0)[1][2][3].

It is known that elemental Se in the redox state of zero is generally considered to be biologically inert. However, there is one kind of nano red selenium (Nano-Se) that was demonstrated to have lower short-term toxicity and keep similar bioavailability compared with selenite.

Recent studies show that Selenium supplementation enhanced the element concentration in blood and seminal fluid, but did not change the spermatozoal quality characteristics in subfertile men[4]. Selenium has certain effects on animal reproductive organs fertility and indirect effect for a neural system[5].Little is known, however, about the regulation of Selenium on neurons, the molecular mechanism underlying Se toxicity is still not completely understood. In studying the effects of Selenium on ion channels, we have already observed that sodium selenite modulated the TTX-S voltage-gated sodium ion current in dose-dependent and time-dependent way on DRG cells.

In the present study, the isolated rat dorsal root ganglion neurons were used to elucidate the effects of Nano-Se on TTX-S voltage-dependent Na^+ channels by the whole-cell patch-clamp technique in attempts to clarify whether Nano-Se modulates neuron cell Na^+ currents and analyze the detailed action of Nano-Se on Na^+ channels.

II. Materials And Methods

A. Isolation of Dorsal Root Ganglion Neurons

2-3-week-old Sprague-Dawley rats, irrespective of sex, were decapitated, and the thoracic and lumbar segments of vertebrate column were dissected and longitudinally divided into two halvies along the median lines on both dorsal and ventral sides. The DRGs together with dorsal and ventral roots and attached spinal nerves were taken out from the inner side of each half of the dissected vertebrate and transferred into Dulbecco's Modified Eagle's Medium (DMEM, Sigma) at pH=7.4.After the removal of attached nerves and surrounding connective tissues, the DRGs were minced with iridectomy scissors and incubated with enzymes including trysin (type III, Sigma) 0.5mg/ml, collagenase (type IA, sigma) 1.0mg/ml and DNase (type IV, sigma) 0.1mg/ml in 5ml DMEM at 35^0C in a shaking bath for 40 min. To stop the enzymatic digestion 1.25mg/ml soybean trypsin inhibitor (type II-S1, Sigma) was added. The isolated neurons were transferred into a 35-mm culture dish and kept still for at least 30 min. All experiments were performed at room temperature (20-30^{o}C)[6][7][8].

B. Solutions and Drugs

The external solution contained (in mM) N_aCl 150, $CdCl_2$ 2.5 M_gCl_2 1, HEPES 10, D-glucose 10, its osmolarity was adjusted to 340mOsm with sucrose and pH was adjusted to 7.4 with N_aOH. In voltage-clamp experiments, the patch-pipette (internal) solution contained (in mM) NaCl 12, CsCl 140, $CdCl_2$ 1, $MgCl_2$ 2, EGTA 11, HEPES 10, ATP 2, the pH was adjusted to 7.4 with CsOH and the osmolarity was adjusted to 310 milliosmol. Solution of selenium was prepared daily in external solution, and selenium was added as Nano-Se.

C. Electrophysiology Experiments

Current and voltage clamp experiments utilized the whole- cell recording configuration with a EPC-9 patch clamp amplifier(made in Germany). Pipettes had resistances

of 2-5MΩ.Series resistances and junction potential were compensated. Membrane currents were filtered at 5kHz and sampled at 0.5-5ms intervals.

D. Statistical Methods

Data analysis is based on IGOR Pro 5.03 (WaveMetrics, Inc) and Clampfit 9.0, All data are presented as mean±SEM. Statistical analyses were performed using Student's unpaired and paired t tests and $P<0.05$ was defined as significant.

III. RESULTS

A. Nano-Se Reversibly Decrease the $I_{Na(TTX-S)}$ in Open-Channel Block Manner without Affecting $I_{Na(TTX-R)}$

When a rat dorsal root ganglion cell held at -80mV was given 100ms depolarizing test pulses from -90 to +60mV, a series of evoked TTX-S Na^+ currents were recorded. The currents were judged to be TTX-S type when the current decay was accomplished within 5ms during a depolarizing pulse of -30mV. All the TTX-S Na^+ channels could be blocked by 1μmol/L TTX (Fig.1A).

The current/voltage (I/V) relationship was obtained (Fig.1B). The current was activated at -60mV, reversed at 61.34±0.021mV, and the average peak current was 2.13±0.21nA(n=8). About 3 min after perfusion with 0.01mM Nano-Se containing solution, the activation threshold potential of current changed from –60mV to –70mV, the peak current was decreased significantly to 1.72±0.31nA($P<0.05$) and the reversal potential was slightly shifted to 56.78±0.054mV. From the I-V relationship, the peak currents did not change a lot in hyperpolarizing test pulses before and after the application of Nano-Se, it is indicated that inhibitor effect of Nano-Se is open–channel block, that is to say, Nano-Se can decrease the sodium currents only when the channels are open.

The G/V curves were computed from g/g_{max}, where $g=I/(V-V_{rev})$ is the conductance at test voltage V, I is the current amplitude, V_{rev} is the reversal potential for sodium ion, and g_{max} is the maximum conductance. The curves were fit with the Boltzmann equation:

$$g/g_{max} = 1/\{1+\exp[(V_{g0.5}-V)/k]\}$$

where $V_{g0.5}$ is the potential at which g/gmax reaches its half-maximal value and k is the slope factor. The curve (Fig.1C) was shifted in the hyperpolarizing direction by Nano-Se. The $V_{g0.5}$ was shifted from -16.578±0.752mV to -24.188±2.8mV(n=8 $p<0.01$),which is significantly different from the spontaneous shift of –1.9±0.6mV(n=6,$p<0.001$). In the effect of Nano-Se, the slope factor k changed from 6.9166±0.656 to 21.845±3.33 (n=8$p<0.01$). The prohibitive effect of Nano-Se was reversible, With 8 min of wash in Selenium-free solution, the decreased currents were recovered. An example of the time course of the change in the peak of I_{Na} is shown in Fig.1D.

The rat dorsal root ganglion neuron cells used in this study expressed both tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) Na^+ channels. I_{Na} recorded at a test potential of -30mV from a holding potential of -80mV, in the presence of 1μmol/L TTX, was TTX-R sodium current. In fact, this current was not significantly modified by Nano-Selenium (n=5, data not shown).

The prohibitive effect of Nano-Se is concentration-dependent manner. Nano-Selenium decrease the TTX-S I_{Na} in a concentration-dependent manner. Fig.1E shows the dose-response relationship. Data points were fitted to the Hill equation, that is:

$$\text{Inhibition}(\%)=[\text{drug}]^n E_{max}/([\text{drug}]^n+[EC_{50}]^n)$$

where E_{max} is the maximum enhanced effect, n is the Hill coefficient, EC_{50} is the concentration for half-maximal effect of drug. E_{max}=110.62±8.3, n=0.5059±0.101, EC_{50}=86.266 ±3.4 (n=8, $p<0.01$).

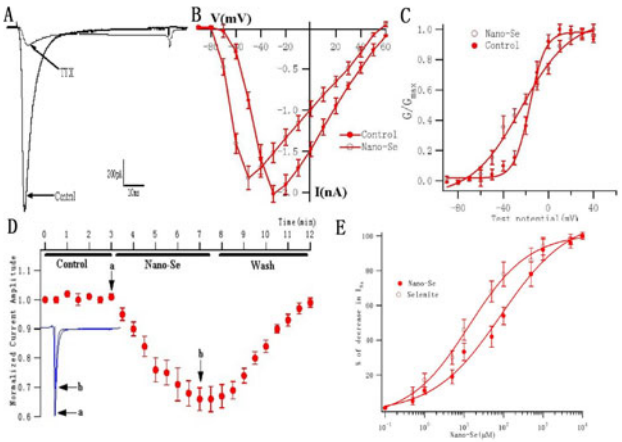

Fig. 1. The effect of Nano-Se on Na^+ current in rat dorsal root ganglion neurons. (A) The Na^+ currents could be completely blocked by TTX. (B) Current/voltage relationships taken before and after application of 0.01mM Nano-Se (n=8). The data were expressed as mean±S.E.M. (C) The conductance – voltage (G–V) relationship curves. The curves were fit to the Boltzmann equation (see text), o.o1mM Nano-Se for 4 min, g, conductance; g_{max}, maximum conductance.(D) One example to show time course of the effect of Nano-Se, I_{Na} was measured as peak current at a test potential of -30mV. After I_{Na} was stabilized, the application of Nano-Se decreased the current from 2.28nA (a) to 1.72nA (b) After 5 min of washing, the current was 2.26 nA.(E) Concentration-response curve of Nano-Se on I_{Na} in rat dorsal root ganglion neurons. Data points represent the average percentage of current decrease after Nano-Se application. Points were curve-fit using the Hill equation. The data were expressed as mean±S.E.M(n=8) .

B. Nano-Selenium Shifted the Steady-State Activation and Inactivation Curves for I_{Na} to More Negative Potentials

The I/V curves in Fig.1 show that not only the peak current was decreased by Nano-Se, but also the potential giving maximal I_{Na} was shifted to the left from -30 to -50 mV, with the change in the activation threshold. The negative shift of I_{Na} peak observed after addition of 0.01mM Nano-Se was further studied with regards to the analysis of both steady-state activation and inactivation parameters.

The superimposed curves in Fig.2 show that both activation and inactivation relationships are shifted to the left in the presence of 0.01mM Nano-Se. Data points were fitted to the Boltzmann function.

The data for activation and inactivation parameters, calculated from the Boltzmann function by fitting to the curve in Fig.2, were summarized in Table1. A comparison of these pooled data confirms that the half-maximum voltage for activation or inactivation, V_h is always significantly($P<0.05$) shifted to the negative direction by the addition of Nano-Selenium. This shift is achieved without change in the slope factor k for activation curves but with a

significant (P<0.05) decrease of k for inactivation curves.

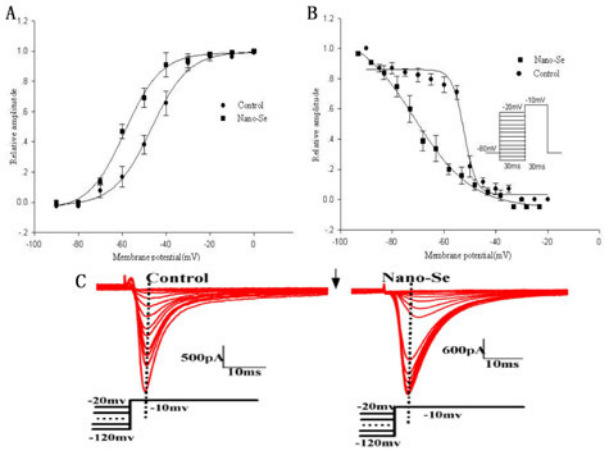

Fig. 2. The effect of Nano-Se on both steady-state activation (dα) and inactivation (fα) curves in rat dorsal root ganglion neurons. (A) The dα curves of I_{Na} before and after application of Nano-Se (n=8). (B) The fα curves of I_{Na} before and after application of Nano-Se(n=8). Pulse protocol is given in the inset, The data for the steady-state kinetic parameters were both fitted to the Boltzmann equation. The data were expressed as mean±S.E.M.(C) The inactivation sodium currents before and after application of Nano-Se.

TABLE I
EFFECTS OF NANO-SE ON STEADY-STATE ACTIVATION AND INACTIVATION PARAMETERS OF I_{NA}

	Activation		Inactivation	
	V_h(mV)	K	V_h(mV)	K
Control (n=8)	-46.8	8.5	-52.3	2.3
Nano-Se(0.01mM n=8)	-58.7	8.2	-68.1	11.1

Values of Vh and K were obtained from data in Fig.3 fitted by Boltzmann function.

C. Recovery from Inactivation

The Na+ channels recovered from inactivation. The time required for the recovery could be tested by a dual-pulse protocol, as illustrated in Fig. 3*A*. The dual pulse included two 10 ms pulses; the first prepulse was from a holding potential of -80 mV to -10 mV, then the second (test) pulse was also from -80mV to -10 mV and finished at the holding potential. The recovery was defined as the percentage of the Na+ current induced by the second pulse versus that induced by the first pulse. The interval between the pre- and test-pulses was varied. The cells were held at given holding potentials for > 5 ms between dual-pulse stimulations, so that the Na+ channels reached steady state before the next dual-pulse stimulation. As shown in Fig. 3*A*, while the Na+ current induced by the first pulse was the same for all dual-pulse stimulations, the Na+ current induced by the second pulse was strongly dependent on the interpulse time. Surprisingly, the recovery was much slower in the presence of Nano-Se than absence of it. The recovery from inactivation was a function of the pulse interval(Fig.3B), The recovery curves were well fitted by single exponentials:$P_2/P_1 = y_0+a*(1-e^{-bx})$，recovery time constant is τ =1/b. In the absence of Nano-Se, y_0=-0.2768±0.04,a =1.2073±0.0367,b=0.0295±0.0016（n=8,p<0.001）, τ =1/b= 34ms; However, in the appearance of Nano-Se, y_0=-0.1867±0.0327,a=1.1388±0.0279,b=0.0208±0.0013（n=8,p<0.001）, τ =1/b= 48ms.

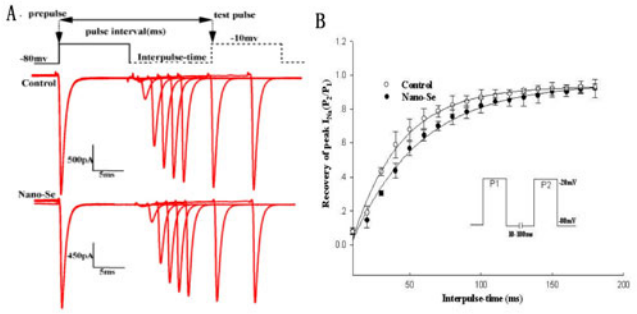

Fig. 3. Fig.3A is the time course of recovery of the Na^+ current from inactivation. The dual-pulse protocol is shown in the top trace. By changing the pulse interval time, a series of dual pulse-induced currents were superimposed according to pulse time. Fig.3B, the recovery time course. The recovery time constants was slower in the application of 0.01mM Nano-Se.

D. Interaction between the Effects of Nano-Se and Na_2SeO_3 at the I_{Na}

Fig.4 shows the interactions of Nano-Se and Na_2SeO_3 at the I_{Na} in rat dorsal root ganglion neurons. When the cell was treated with 0.01mM Nano-Se alone, the peak current was decreased by 26±4%(n=4). Further addition of 0.01mM Na_2SeO_3 didn't caused a further decrease of the current (Fig.4A n=4). Meanwhile, in the experiments that the cells were treated with 0.01mM Na_2SeO_3 first and then 1nM Nano-Se(Fig.4B), we found that the current was firstly decreased by 57±5% and then keep the value without any change. It was suggested that Na_2SeO_3 lost the ability to decrease the current under the condition of application Nano-Se firstly (n=4).

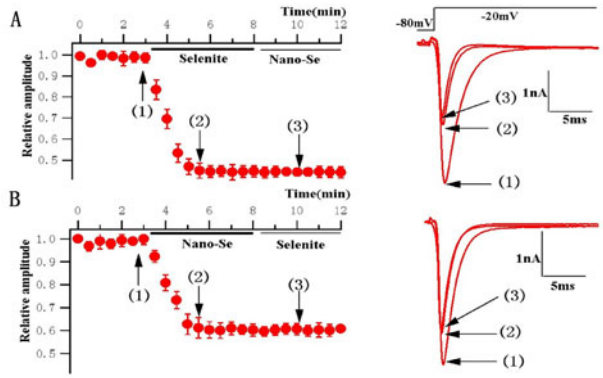

Fig. 4. Interaction of Nano-Se and Na_2SeO_3 in DRG cells. Superimposed traces were elicited by a depolarizing test pulse to -20mV from a holding potential of -80mV. Time courses of the effect of the drugs on Na^+ channels were shown. (A) After the I_{Na} was elicited by 0.01mM Na_2SeO_3, Nano-Se lost the ability to cause an decrease in the current. (B) After the I_{Na} was inhibited in the application of 0.01mM Nano-Se, the peak current was maintained without any change in the effect of 0.01nM Na_2SeO_3. The number in brackets shows the sequence to take the trace.

E. Digression on the Amplitudes of Current Fluctuations

By using the variability in current measurements, we can calculate how many channels are contributing to the macroscopic current and the value of the single ion currents. The nonstationary variance-mean method made the macroscopic kinetic descriptions are replaced by microscopic state diagrams. The function of variance-mean parabola is that:

$$\sigma_x^2 = \frac{1}{n}\sum_{i=1}^{n}(x_i - \bar{x})^2 \quad \sigma_I^2 = Npi^2 - Np^2i^2 = iI(1-p) = iI - I^2/N$$

σ_x^2 is the variance, $x_1x_2x_3x_4$..... x_n is the observation, i is the single channel current, N is the the number of the channels. Using the variability in current measurements, we calculated that the number of the channels contributing to the macroscopic current N=924, single ion current i = 5.64pA(Fig.5A). After the application of 0.01mM Nano-Se, N=588, i = 5.49pA(Fig.5B). The result demonstrated that Nano-Se decrease the sodium current in microscopic state.

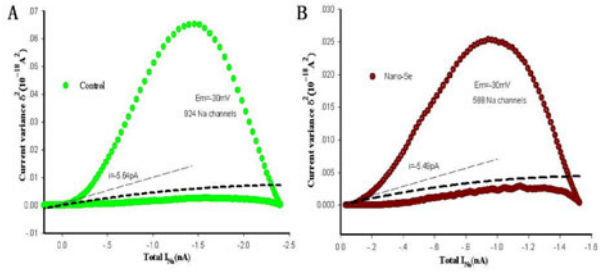

Fig. 5. Nonstationary Variance-Mean Parabola.

IV. DISSCUSIONS

In the present study, Nano-Se, as a Nano-technology medicine, was demonstrated to induce TTX-S voltage-gating Na^+ channel current decline without affecting the TTX-R Na^+ current, shift the maximum of I/V relationship towards more negative potential with affecting activation threshold potential of the current. Thus, the effect of Nano-Se on I/V relationship might be the consequence of the hyperpolarizing shift in the activation curve. Nano-Se shifted the steady-state activation and inactivation curves to more negative potential and caused a significant increase in the slope of inactivation, which reflects a jield of less current for a given potential. These results indicate that Nano-Se may alter the voltage sensitivity of the channel. Meanwhile, Nano-Se also affect Na^+ channel activation kinetics over a wide range of membrane potentials, It indicates that Nano-Se affected the transition from the closed to the open state of the channel. Additionally, the recovery time constant is larger in the presence of Nano-Se, possibly reflecting a longer time is needed for recovery from inactivation state.

In the contrast with Na_2SeO_3, Nano-Se also have the ability to effect the TTX-S Na^+ current in dose-dependent way, whereas Nano-Se might have a weaker inhibitory effect on I_{Na} compared with marked decrease caused by selenite. These results indicated that both Nano-Se and sodium selenite were neurotoxic in short term and large dose treatment.

At present, the molecular mechanism underlying Se toxicity is still not completely understood. Our present results demonstrated that Nano-Se had similar bioavailability and lower acute toxicity in the term of modulation effect of I_{Na} in DRG cells.

TTX-S Na^+ channel, as well as other voltage-dependent ion channel, can be regarded as pharmacological receptors containing discrete drug binding sites. Previous study of Selenium showed that 0.01mM Na_2SeO_3 was a potent inhibitor of TTX-S Na^+ ion in DRG cells. Here, we examined the interactions of 0.01mM Na_2SeO_3 and 0.01mM Nano-Se directly at TTX-S Na^+ channels in rat dorsal root ganglion neurons. We obtained that the DRG cells pretreated with 0.01mM Na_2SeO_3 didn't preserve the ability to respond to further decline in the effect of 0.01mM Nano-Se. At the same time, we didn't found significant decreased change of Na^+ currents with 0.01mM Na_2SeO_3 after the cell was pretreated with 0.01mM Nano-Se. Due to the results, it is conceivable that Nano-Se and selenite acted in different bingding sites, there may be a negative allosteric interaction between the Nano-Se binging site and the selenite binging site. However, another hypothesis is that both of them effect on a competitive binding site, The combining between the first drug and the binding site is able to initiate the oxidation of proein thiol groups, Reaction with thiols could alter activity of many different essential sulfhydry-containing enzymes and ion channel protein structure. However, owing to the structural complexity of the ion channels and their ability to exist in various closed, opened and inactivated states, interaction of drugs at ion channels are sometimes complicated.

The results in this study demonstrate that Nano-Se, as a new Nano-technological product, has bioavailability. It can modulate TTX-S voltage-dependent Na^+ channel currents in rat dorsal root ganglion neurons, and indicate that Nano-Se possesses ability in regulating neuron cells. This finding brings us more interesting in studying this Nano-medicine. If this drug is valuable in medical use in the future, its effect on nervous system diseases can't be ignored.

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