Relative Reactivity of Dihydropyridine Derivatives to Electrogenerated Superoxide Ion in DMSO Solutions: A Voltammetric Approach

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Purpose. To evaluate the reaction of a large series of pharmacologically significant 1,4-dihydropyridine (1,4-DHP) compounds with superoxide ($O_2^{\cdot-}$) in dimethylsulfoxide using differential pulse voltammetry and controlled potential electrolysis.

Methods. Differential pulse voltammetry was used to track the consumption of $O_2^{\cdot-}$, and controlled potential electrolysis was used to electrogenerate $O_2^{\cdot-}$.

Results. With the addition of 1,4-DHP, the oxidation peak current of $O_2^{\cdot-}$ decreased concentration dependently, suggesting that 1,4-DHP reacts with $O_2^{\cdot-}$, that is, 1,4-DHP scavenges $O_2^{\cdot-}$ in dimethylsulfoxide.

Conclusions. A very easy and direct voltammetric procedure to study the relative reactivity of different 1,4-DHP with $O_2^{\cdot-}$ is proposed. Using the proposed method we have found that all commercial 1,4-DHP reacts with $O_2^{\cdot-}$. The following order of rates was obtained: felodipine ≥ vitamin E > isradipine > nimodipine > furnidipine > nitrendipine > nisoldipine > nifedipine. Furthermore, it was demonstrated that the hydrogen at the N-position of 1,4-DHP compounds could be released as a proton in the presence of $O_2^{\cdot-}$, thus the electrogenerated $O_2^{\cdot-}$ worked as a proton acceptor to 1,4-DHP.

KEY WORDS: differential pulse voltammetry; superoxide; 1,4-dihydropyridine; scavenger.

INTRODUCTION

Oxygen metabolism has attracted an increasing interest all over the world. Superoxide radical anion, $O_2^{\cdot-}$, is the one electron reduction product of oxygen and is biologically generated by relevant sources from autoxidizable small molecules such as catecholamines to whole cells such as neutrophils, monocytes, and macrophages (1). This radical can be induced by electron transfer reaction *in vivo* to generate other reactive oxygen species such as hydroxyl free radical and hydrogen peroxide. Thus, the $O_2^{\cdot-}$ is highly reactive and toxic and can cause oxidation of biomacromolecules as well as initiate radical-chain oxidation in tissues. For this reason, substances that can react rapidly with $O_2^{\cdot-}$ are of major importance among antioxidants. Interest in antioxidants is growing constantly, but many problems are not resolved, mainly focused on methods of determining antioxidant capacity. Generally, these methods are indirect and are based on inhibition of a model oxidation reaction in solution by the antioxidant following a control signal by an adequate technique. Consequently, there is a continuing effort to develop methods suitable for screening antioxidant activity by directly following the interaction between $O_2^{\cdot-}$ and an antioxidant molecule.

Previous studies (2–9) have suggested that 1,4-dihydropyridine (1,4-DHP) derivatives provide an antioxidant protective effect that may contribute to their pharmacologic activity. Thus, this type of drug, in addition to its well-known effect on calcium metabolism, could itself have an antioxidant effect. Specifically, we have found recently (10) that nisoldipine, one of these 1,4-DHP compounds, reacts directly with $O_2^{\cdot-}$, the latter acts as a Brönsted base deprotonating nisoldipine, and nisoldipine acts by scavenging $O_2^{\cdot-}$.

The reduction of oxygen to superoxide anion in aprotic solvents has been known since 1965 (11–15). Considering this antecedent with the fact that membrane accumulation of dihydropyridines may occur because of their lipophilic nature (4), it is of great interest to study the reactivity between dihydropyridines and $O_2^{\cdot-}$ in aprotic media.

Recently, Korotkova and co-workers (16) have described a method for studying the antioxidant activity of some compounds using the diminution of the electrochemical reduction current of oxygen after the compounds had reacted with superoxide. In this article, we propose an alternative method: studying the electrochemical oxidation current of superoxide directly instead of the oxygen signal. Consequently, this article reports a study of the reactivity of 1,4-DHP derivatives with $O_2^{\cdot-}$ in aprotic media for establishing the relative scavenger properties of these drugs.

MATERIALS AND METHODS

Drugs (Fig. 1)

All drugs were 100% chromatographically pure:

Nisoldipine: 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid methyl 2-methylpropyl ester (Laboratorio Chile, Santiago, Chile)

Nifedipine: 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (Laboratorio Mintlab, Santiago, Chile)

Nimodipine: 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid, 2-methoxyethyl 1-methylethyl ester (Laboratorio Saval, Santiago, Chile)

Furnidipine: 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine-dicarboxylic acid, methyl 2-tetrahydrofuryl-methyl ester (Laboratorio Alter, Madrid, Spain)

Nitrendipine: 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid, ethyl methyl ester (Laboratorio Sanitas, Santiago, Chile)

Isradipine: 4-(4-benzofurazanyl)-1,4 dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid, methyl 1-methylethyl ester (Laboratorio Sandoz, Santiago, Chile)

Felodipine: 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid, ethyl methyl ester (Laboratorio Saval, Santiago, Chile)

Compound I: 1-ethyl-1,4-dihydro-2,6-dimethyl-4-(4-methoxyphenyl)-3,5-pyridinedicarboxilic acid dimethyl ester

Reagents

The aprotic solvent dimethylsulfoxide (DMSO) used in all experiments was purchased from Merck (Darmstadt, Ger-

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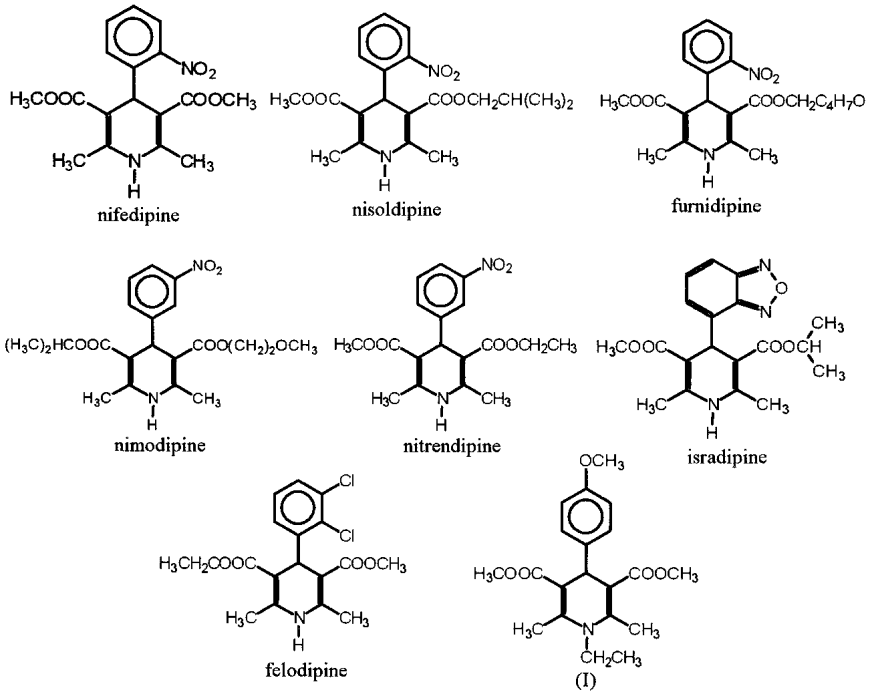

Fig. 1. Chemical structures of aryl 1,4-dihydropyridine derivatives.

many) and was dried with 3-Å molecular sieves. All the electrochemical experiments were carried out in aprotic media (100% DMSO) with 0.1 M tetra-butylammonium perchlorate (TBAP) purchased from Fluka (Buchs, Switzerland). Oxygen (99.8% pure) and nitrogen (99.9% pure) were purchased from AGA (Santiago, Chile).

Potassium superoxide (KO_2) (97% pure) was purchased from Sigma (Steinheim, Germany), crown ether/18-crown-6 (> 98% pure) and vitamin E 50% (52% pure) were purchased from Merck.

Potassium Superoxide Solution

A 0.1 M KO_2 solution in dimethyl sulfoxide using crown ether was prepared according to the following procedure: 71.1 mg (1 mmol) of finely powdered KO_2 and 500–800 mg (2–3 mmol) of crown ether/18-crown-6 were placed in a dry flask and 10 ml of dimethyl sulfoxide were introduced into the flask. The mixture was stirred for 0.5–3 h to give a clear pale yellow solution. The solution, stored at –40°C, was stable for several weeks with no apparent decomposition. Different solutions were made from this stock solution.

For calibration plots, a series of solutions containing superoxide concentrations between 5×10^{-4} and 5×10^{-3} M in DMSO with 0.1 M TBAP were prepared and measured by differential pulse voltammetry (DPV).

Controlled Potential Electrolysis

Controlled potential electrolysis (CPE) was carried out using a mercury pool cathode (area 10.18 cm^2). The applied potential (-1000 mV) was obtained using a BAS CV-50 W voltammetric analyzer potentiostat as a source. The CPE was carried out in a two-compartment cell with the counter electrode separate from the pool electrode. In the three-electrode system, an Ag | AgCl | NaCl(sat) electrode was used as the reference electrode. Before each run, the solutions were first degassed with nitrogen and then saturated with oxygen. The solution was stirred continuously during the electrolysis. Nitrogen was continuously passed over the solution during the electrolysis and then was passed through the solution to purge all possible nonreacted dissolved oxygen. CPE was tracked by the DPV method for proving the generation of superoxide radical anion. Nitrogen was continuously passed over the solution during the DPV experiment.

The concentration of superoxide anion generated by controlled potential electrolysis of an oxygen-saturated solution in DMSO was determined using a calibration curve constructed with $O_2^{\cdot-}$ chemically generated (KO_2) as a reference standard.

Apparatus and Procedures

DPV experiments were performed with a BAS CV-50 W voltammetric analyzer. All measurements were carried out in a three-electrode measuring cell. A glassy carbon disc electrode with area of 0.071 cm^2, a platinum wire counter electrode, and an Ag | AgCl | NaCl(sat) reference electrode were used for the measurements. All voltammograms were carried out at a constant temperature of 25°C. The glassy carbon disk working electrode was polished using successively 0.3 and 0.05 μm alumina powder on a polishing cloth before each experiment.

RESULTS AND DISCUSSION

It is well known that electrochemical reduction of dioxygen in dimethylsulfoxide yields stable solutions of superoxide ($O_2^{\cdot-}$) (14). Consequently, solutions containing different quantities of $O_2^{\cdot-}$ can be electrogenerated by CPE. On the other hand, we have found that $O_2^{\cdot-}$ produces a very well resolved anodic differential pulse voltammogram in aprotic media due to the one-electron oxidation to produce oxygen. The anodic differential pulse voltammograms of different solutions con-

taining $O_2^{\cdot-}$ obtained from both—electrogenerated from CPE and chemically from KO_2—are shown in Fig. 2. In this experiment, we have unambiguously demonstrated that the observed DPV corresponds to the oxidation of $O_2^{\cdot-}$ obtained as a consequence of an anodic sweep starting at –900 mV and finishing at –400 mV with a potential peak of –720 mV. Special precaution must be taken in eliminating all the O_2 present in the superoxide solutions, since this interferes with the DPV peak of $O_2^{\cdot-}$. The oxygen present in solutions can be properly eliminated by bubbling with nitrogen. On the other hand, the superoxide cannot be eliminated from the solutions with nitrogen. In Fig. 3a, we can observe a DPV obtained from a solution containing only the supporting electrolyte in DMSO before and after eliminating the O_2, proving that O_2 dissolved in solution also produces an anodic DPV capable of interfering with the $O_2^{\cdot-}$ signal. This is explained by the fact that at the starting potential (–900 mV), O_2 is in the reduced form ($O_2^{\cdot-}$) and consequently can be oxidized in the DPV run. On the other hand in Fig. 3b, we can observe the null effect of bubbling nitrogen in a solution containing only $O_2^{\cdot-}$. Furthermore, we have found that the peak current of the DPV peak varies linearly with the $O_2^{\cdot-}$ concentration (Fig. 4), obtaining a calibration curve described by the equation $i(\mu A) = 4.829C(mM) - 0.873$ ($r = 0.9978$).

The reproducibility of the $O_2^{\cdot-}$ oxidation peak was tested with ten independent runs, obtaining a variation coefficient of 4.8%. To check the accuracy and precision of the developed method, we carried out a recovery study obtaining 96.8% ± 5.4 for ten independent solutions containing 1×10^{-3} M of KO_2. According to these studies, the developed method appears to be adequately reproducible, accurate, and precise for determining $O_2^{\cdot-}$ concentration in solution.

To check the stability of the electrogenerated $O_2^{\cdot-}$ solutions by CPE in DMSO, we have used DPV to follow the time course of the $O_2^{\cdot-}$ natural decay in this medium. Figure 5 shows the DPV obtained following the natural decay of $O_2^{\cdot-}$ after CPE generation. Figure 5e shows a DPV of a solution without $O_2^{\cdot-}$ to obtain the residual current value (i_{res}). As a conclusion of this experiment, we can affirm that the $O_2^{\cdot-}$ oxidation peak current remains stable until approximately 20

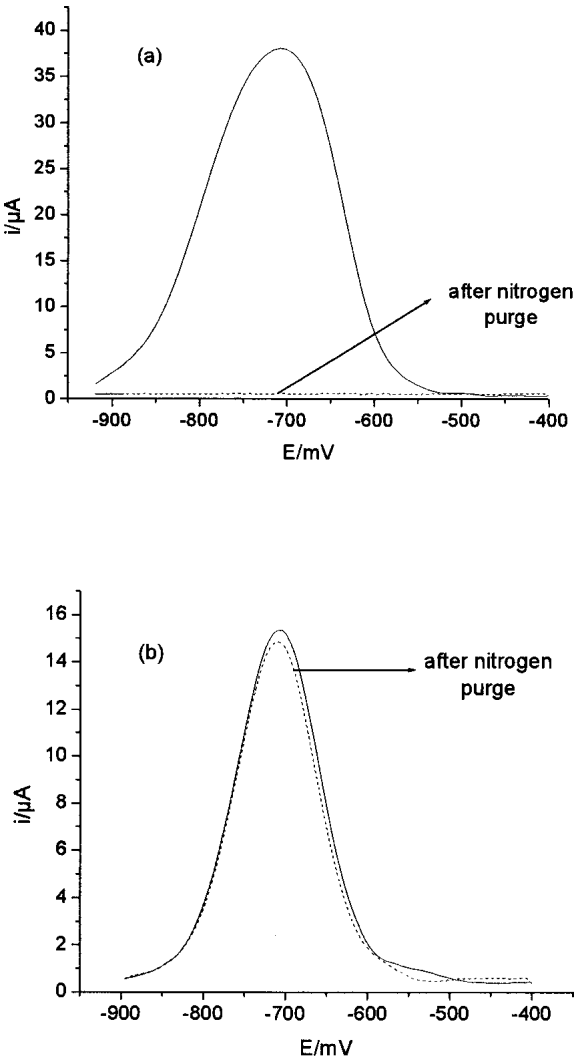

Fig. 3. DPV response before and after the solution was purged with nitrogen: (a) an oxygen-saturated solution; (b) an electrogenerated superoxide solution.

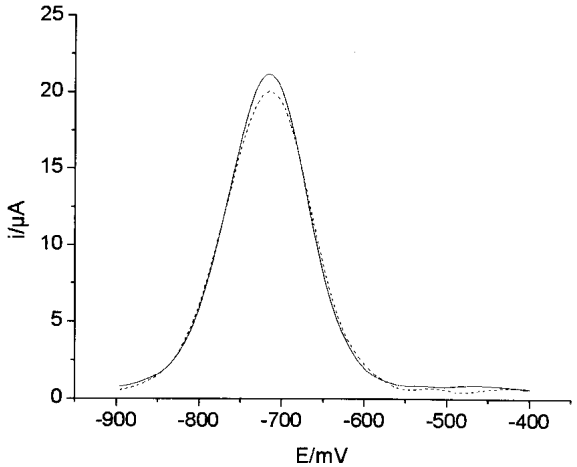

Fig. 2. DPV of superoxide solutions obtained from: (solid line) 3mM KO_2, 0.1M TBAP in DMSO, and (dashed line) $O_2^{\cdot-}$ generated after 10 min of CPE in saturated oxygen solution containing 0.1 M TBAP in DMSO.

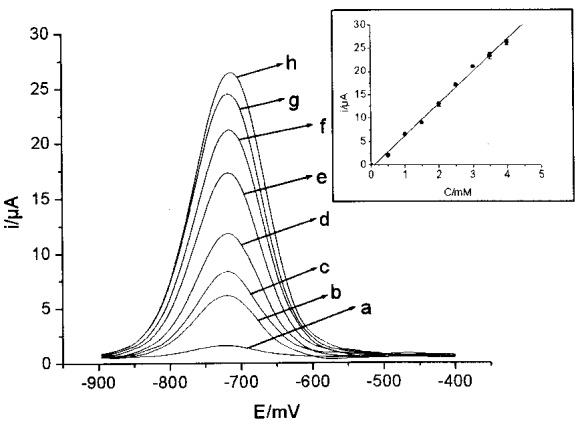

Fig. 4. DPV of different solutions of KO_2, 0.1M TBAP in DMSO: (a): 0.5 mM; (b):1 mM; (c): 1.5 mM; (d): 2mM; (e): 2.5mM; (f): 3 mM; (g): 3.5mM; (h): 4mM. Insert: calibration curve for KO_2.

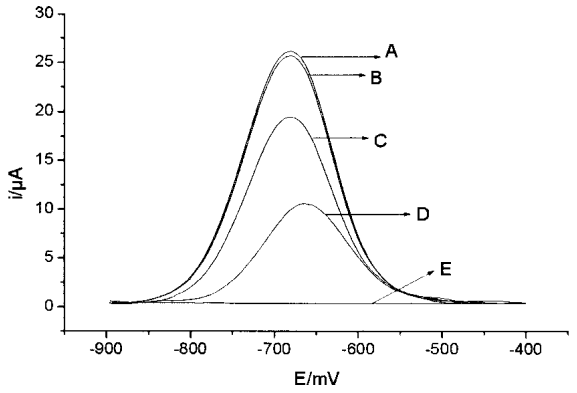

Fig. 5. Differential pulse voltammograms of superoxide anion solutions in 0.1M TBAP DMSO at different times after generation by CPE. (A) 0 min; (B) 15 min; (C) 60 min; (D) 120 min ; Curve E shows the DPV of a solution without superoxide.

min after generation, enough time for studying the interaction with 1,4-DHP.

Surprisingly, it was found that the addition of 1,4-DHP to a solution containing the electrochemically generated $O_2^{\cdot-}$ produced a notorious diminution in the peak current of the $O_2^{\cdot-}$ DPV peak. This can be ascribed to an interaction between $O_2^{\cdot-}$ and 1,4-DHP. In fact, 1,4-DHP, reacting with the $O_2^{\cdot-}$, decreased the concentration of $O_2^{\cdot-}$ at the electrode, thus decreasing the peak current of the DPV. Consequently, the anodic peak current due to the $O_2^{\cdot-}$ oxidation decreased and therefore can be used as a comparative value of the reactivity with $O_2^{\cdot-}$ in the solution being analyzed. It should be noted that all the 1,4-DHP being studied is not adsorbed on the surface of the glassy carbon electrode in the potential range of the $O_2^{\cdot-}$ oxidation.

To investigate the relative reactivity of 1,4-DHP compounds with $O_2^{\cdot-}$, anodic voltammograms of $O_2^{\cdot-}$ in supporting electrolyte containing different concentrations of 1,4-DHP under investigation were recorded. In Fig. 6, we can observe the diminution in the peak current of the DPV due to $O_2^{\cdot-}$ oxidation as a consequence of adding increasing concentrations of nisoldipine, a representative 1,4-DHP dihydropyridine. All the studied 1,4-DHP showed the same behavior, that is, the $O_2^{\cdot-}$ oxidation peak current diminished as the 1,4-DHP derivative concentration increased.

The 1,4-DHP concentrations varied from 1×10^{-3} M to 1×10^{-2} M. The oxidation signal diminished immediately when the dihydropyridine derivative was added, suggesting that these substances react with $O_2^{\cdot-}$ before its natural decay, exerting a protective effect as antioxidant drugs. It should be noted that all the studied drugs were electroinactive in the potential zone where superoxide produces an oxidation signal. When the 4-nitroaromatic substituted 1,4-DHP are reduced to the corresponding nitro radical anion at potentials more cathodic than –1000 mV, they are nonreduced at the initial potential of the anodic sweep.

Curves of the relative change in the superoxide oxidation current ($i/(i_{O2^{\cdot-}} - i_{res})$) against dihydropyridine concentrations were obtained. All these curves show an exponential decay. To obtain a linear behavior, we have displayed the logarithmic analysis of these curves. Some of the 1,4-DHPs being tested are shown in Fig. 7. The slopes of these lines are suggested to be coefficients of the superoxide scavenger activity for these drugs. In Table I, we have included the obtained values for the coefficients named as a relative constant for the interaction between $O_2^{\cdot-}$ and the corresponding 1,4-DHP.

Although the studied compounds are not so different structurally, the method let us compare the $O_2^{\cdot-}$ reactivity of this type of drugs. As can be seen, felodipine and isradipine had the greatest reactivity of the group and have comparable reactivity with the well-known antioxidant vitamin E.

In a previous study (10), it was demonstrated that the hydrogen at the N-position of nisoldipine could be released as a proton in the presence of $O_2^{\cdot-}$ and consequently act as a scavenger of $O_2^{\cdot-}$. In the present case, we have assumed a similar effect with all other members of the family, and consequently, the scavenging of $O_2^{\cdot-}$ can be due to the hydrogen at the N-position. In the case of vitamin E, the observed reactivity with $O_2^{\cdot-}$ can be explained by the interaction of the phenolic hydrogen with $O_2^{\cdot-}$. Furthermore, there are many published examples of the interaction of a relatively acidic hydrogen in a molecule with superoxide in aprotic medium. A

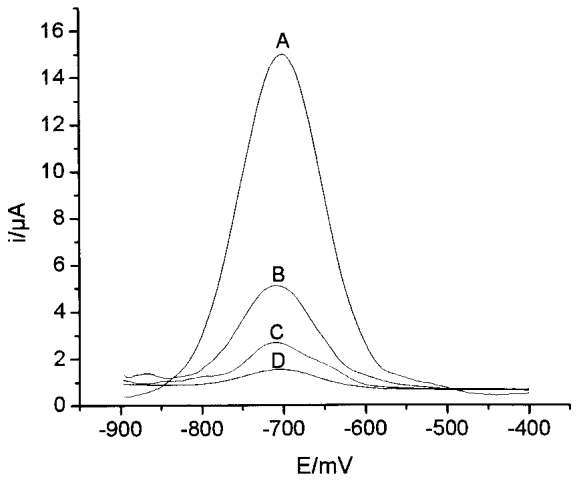

Fig. 6. Voltammograms of a 3.2×10^{-3} M electrogenerated superoxide solution in the presence of different nisoldipine concentrations. (A) without nisoldipine; (B) 3 mM; (C) 7 mM; (D) 10 mM.

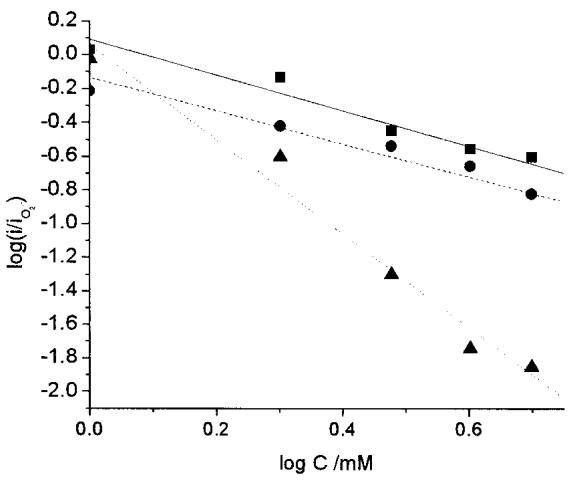

Fig. 7. Curves of the relative change of the superoxide oxidation current against 1,4-DHP concentration.(●) nisoldipine; (■) nitrendipine; (▲) felodipine.

Table I. Scavenger Activity Coefficients for the Drugs in Study

Compounds	Relative constant	SD
Felodipine	2.86	0.16
Vitamin E	2.79	0.25
Isradipine	2.59	0.16
Nimodipine	2.12	0.11
Furnidipine	2.06	0.09
Nitrendipine	1.34	0.04
Nisoldipine	0.84	0.04
Nifedipine	0.73	0.03

very recent published example concerns indole compounds bearing a hydrogen at the N-position (17). In these indole compounds, the hydrogen at the N-position acted as a proton donor to $O_2^{\cdot-}$. To probe the hypothesis that the hydrogen at the N-position in 1,4-DHP compounds is crucial for the interaction with $O_2^{\cdot-}$, we have synthesized a derivative (named as compound I in Fig. 1) in which the hydrogen at the N-position was replaced by an ethyl group. When compound I was added to a solution containing electrogenerated superoxide, no diminution of the oxidation peak current of superoxide was observed, showing that this 1,4-DHP derivative does not scavenge superoxide. Consequently, this experiment strongly supports our hypothesis in the sense that hydrogen at the N-position is crucial in scavenging superoxide.

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