Inhibition of Excitation-Contraction Coupling by a Ca Channel Blocker Nicardipine at Low Temperature in Frog Twitch Fibers

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Abstract Effects of a Ca channel blocker, nicardipine, on excitation-contraction (E-C) coupling were investigated in single twitch fibers dissected from short skeletal muscles of the frog, Rana japonica. The treatment with 20 µM nicardipine at 25°C evoked a reversible twitch potentiation. By contrast, the treatment at the same concentration at 5°C for 30 min evoked an intense twitch inhibition. The inhibition was irreversible. This paralyzed fiber remained at nearly normal resting and propagated action potentials. The treatment with 30 µM nicardipine for 14 min at 6°C completely inhibited a potassium contracture by 190 mEq/l potassium ion for 1.5 h after the removal of nicardipine despite the retention of normal resting potentials. The paralyzed fiber which was previously treated with 20 µM nicardipine for 30 min at 6°C, also remained at normal action potential and at partial potassium contracture, and responded to 3 mM caffeine stimulation with a normal contracture. Dose-dependence curves obtained from the data on the treatments with nicardipine at various concentrations for 30 min at 6°C consisted of an opposite sigmoidal shape with both threshold and half maximum inhibition at 1 and 1.5 µM for twitch and similarly at 5 and 12 µM for potassium contracture. Temperature-dependence curves obtained from the data on the treatments with 20 µM nicardipine for 30 min at various temperatures consisted of a sigmoidal shape with half maximum inhibition at 15°C for twitch and at 12°C for potassium contracture. A working hypothesis “two component-three state model” for E-C coupling was proposed based on the interpreted mechanism of nicardipine action.

Key words: excitation-contraction (E-C) coupling, skeletal muscle twitch fiber, Ca channel blocker, nicardipine, decoupler.

A process called excitation-contraction (E-C) coupling in skeletal muscles is mystifying. Our knowledge is sufficient for contouring its physiological image and the concepts have been delineated (SANDOW, 1965; OETLIKER, 1982; CAILLÉ et al.,

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1985). However, the nature of the mechanism remains hitherto unknown.

Transverse tubule (T) membrane as well as sarcolemma in the fiber has the ability to produce a propagated action potential (Bastian and Nakajima, 1974). The excitation is also called depolarization (Schneider, 1981). On the other hand, sarcoplasmic reticulum (s.r.) within the cell has the ability to release calcium (Ca) ions into myofibrillar space, responding to excitation (Martonosi, 1984; Endo, 1985). The rise in free Ca$^{2+}$, namely Ca transient, leads to a twitch response (Miledi et al., 1977). In a narrow sense, we can consider E-C coupling as the process intervening between T membrane depolarization and the resultant s.r. Ca release.

This paper concerns an experimental result revealing fundamental effect of nicardipine on E-C coupling. This dihydropyridine is a sort of Ca antagonist or Ca channel blocker in other organs (Schwartz, 1984) and evokes a potent vasodilation in both cerebral and coronary vessels in mammals (Takenaka et al., 1976; Takenaka and Handa, 1979). In the fast skeletal muscle, the analogs also block voltage-dependent slow Ca channel located at T membrane (Stefani and Chiarandini, 1982; Ildefonse et al., 1985). This slow channel seems not to play an important part in E-C coupling at normal temperature (Gonzalez-Serratos et al., 1982). This finding is also compatible with the fact that Ca$^{2+}$ influx is not a prerequisite to normal contractile activation (Specker et al., 1979). Furthermore, these drugs are almost uneffective on the s.r. Ca channel in skinned fibers (Ishizuka and Endo, 1982), but facilitate its function in fragmented s.r. vesicles (Wang et al., 1984).

We found that nicardipine is a potent inhibitor of E-C coupling or E-C decoupler at low temperature in the fast skeletal muscle. The potency is equal to or larger than that of known decouplers such as dantrolene sodium (Hainaut and Desmedt, 1974). A similar finding is reported in another analog, D-600 (Eisenberg et al., 1983). In D-600, conditions of both low temperature and plasmalemmal depolarization are indispensable in establishing inhibition. This drug may essentially be use-dependent (Hondeghem and Katzung, 1984).

The preliminary form of this paper has appeared elsewhere (Fujino et al., 1984). More recently, the effect of nicardipine on skeletal muscles at about 20°C was reported by other authors (Hatate, 1986).

MATERIALS AND METHODS

Single twitch fibers were dissected from short flexor and extensor of the hind limbs of the frog, Rana japonica. The flexor was of m. lumbricalis brevis digits IV and V (Gaupp, 1896). These muscles which were seldom used in conventional experiments, were in active demand for experiments on a voltage-clamped depolarization contraction (Bolanos et al., 1986). The extensor was of m. extensor brevis medius digit III. The frog was stocked at room temperature or around 4°C. The frog at room temperature was fed with larvae of the cricket, Gryllus bimaculatus. Unless otherwise stated, the frog stocked at low temperature was used.

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A large difference in electrical and mechanical properties between the flexor and extensor during activities was not detectable. However, we had a general impression that the fibers isolated from the flexor and also from the frog stocked at low temperature were apt to suffer pharmacological actions.

Using a piece of vinyl plate, the isolated fiber was transferred to a homemade muscle chamber (26 × 3 × 2 mm) with 0.16 ml capacity, filled with normal Ringer medium. One tendon was held by a stainless steel hook and the other also by the shaft tip of a tension transducer made of a semiconductor gauge (AE801, AME, Norway). Natural frequency of the transducer was above 240 Hz in water and compliance 0.2 mm/g. This compliance would correspond with 1.5% shortening during a potassium contracture of 244-mg tension development in 3.2-mm-fiber length. After the fiber was stretched to around 120% of the slack length, the following parameters, fiber length, striation spacing, and diameter, were measured with a microscope of 40–800 × magnifications. Medium temperature controlled by a water circulation system was measured with a glass thermister (IBT-2, Ishizuka Electr.).

The composition of a normal Ringer medium was 115 mM NaCl, 2.5 mM KCl, and 1.8 mM CaCl₂ buffered to pH 7.2 with 2.5 mM PIPES. Twitches were evoked by an external electric stimulation of a suprathreshold square pulse with a duration of 0.25 ms. Potassium contractures were induced with a depolarization medium prepared by replacing both NaCl and KCl in the normal medium with 95 mM K₂SO₄. Caffeine contractures were also induced with a caffeine medium prepared by adding 3–15 mM caffeine to the depolarization medium. Preceding application of the depolarization medium, a choline medium prepared by replacing Na⁺ in the normal medium with equimolar choline ions was applied for a short period. This pretreatment eliminated an action potential development triggered with the depolarization medium. Nicardipine hydrochloride (Perdipine) was kindly donated by the Yamanouchi Pharmaceutical Co., Ltd. in Japan and stocked in the aqueous solution at a concentration of 1 mM. The solubility in the normal medium was up to 50 μM.

Resting and action potentials were recorded using flexible microelectrodes (HEISTRACHER and HUNT, 1969) filled with 5 M K-acetate. The microelectrode resistance was about 40 MΩ. In the preliminary experiments, action potentials were alternately recorded from one stretched fiber using a conventional microelectrode filled with either 3 M KCl or 5 M K-acetate, and the difference in shapes of these action potentials could not be discovered. Electrical and mechanical signals were recorded by conventional electrophysiological apparatus and a digital microcomputer system (HP 9825A/S, Hewlett Packard, U.S.A.).

RESULTS

Number of single fibers used was 13. Mean ± S.E. of their length, striation spacing, and diameter were 3.2 ± 0.3 mm (1.9–5.1), 2.5 ± 0.02 μm (2.4–2.6), and
Mean ± S.E. of potassium contracture height and twitch height versus potassium contracture height in normal medium at 6°C in 7 fibers were 3.3 ± 0.2 kg/cm² (2.7–3.9) and 39 ± 3% (31–54), respectively.

Establishment of paralyzed at low temperature

Figure 1 shows effects of nicardipine on twitch responses at different temperatures of the medium in two fibers. The preparations were isolated from one extensor digit III of the well-fed healthy frog stocked at room temperature. Responses of the two fibers were individually obtained using the stimulating and recording apparatus of two channels. The experimental conditions were similar except for one point of difference in medium temperature. The fibers were equilibrated approximately 5.5 h in the normal medium as twitches were synchronously evoked once per min. After this equilibration, the medium was replaced with a normal medium containing nicardipine at a concentration of 20 µM. The fibers were immersed in this medium for 30 min.

A noteworthy change in twitch responses at 25°C was not detectable except for an increased response or potentiation of 140% during the nicardipine treatment (Fig. 2A-b). This observation was similar to that made by other authors (HATAE, 1968) and common to the other analog cases such as diltiazem (GONZALEZ-SERRATOS et al., 1982). Thereafter, we were not concerned with further property of this potentiation.

On the other hand, twitch responses at 5°C diminished gradually and before long became only a trace during the nicardipine treatment (Fig. 1B-b). The fiber was apparently less sensitive to the electric stimulation. The shape of the decay course of the twitch height varied from fiber to fiber. The decay course in this fiber consisted

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of two components with a break point at 7 min. On the other hand, that in such a fiber shown later (Fig. 5) consisted of a singly exponential component with a time-constant of 4.5 min.

Undoubtedly, the diminution in or inhibition of contractility by the nicardipine treatment was dependent on low temperature. By the nicardipine removal, the inhibition still lasted a longer period, where the inhibition even tended to progress further. The twitch responses recovered soon, though very slowly. The twitch inhibition or paralyzation could be called irreversible.

**Retention of electrical ability in paralyzed fiber**

Locating the above noticeable finding upon an E-C coupling concept, we must first allow for a sarcolemmal electrical ability to twitch. Figure 2 shows propagated action potentials recorded from the same fibers as shown in Fig. 1. The initial part of tension development is also shown in the separate trace. The control action potential in the respective fibers was recorded before the nicardipine treatment, and the test action potentials were recorded after the removal of nicardipine. On recording, a microelectrode was impaled at all times in similar positions at each fiber end in the vicinity of the tendon. On the other hand, electrodes for the stimulation were placed near the opposite end.
The first is on an extreme elongation of the control action potential at 5°C (Fig. 2B-a). This elongation arose in the manner of a delayed repolarization phase probably associated with a change in delayed rectification (Nakajima et al., 1962). The half peak width was 11 ms, which was tenfold larger than that at 25°C (Fig. 2A-a). The height was also increased slightly. The observation at low temperature was essentially identical with previous works by other authors (Huxley, 1959; Caputo, 1972b).

The second concerns an influence of the nicardipine treatment. The moderate impression was the case that the propagated action potential of the paralyzed fiber shows nearly a normal waveform (Fig. 2B-b). Strictly speaking, only its height became 91% of the control (Fig. 2B-a). The other parameters such as half peak width and shapes in rise and fall remained almost unchanged. These characterizations still remained in the test action potential at 4 h after the removal of nicardipine (Fig. 2B-c), where the peak of twitch tension recovered 39% of the control (Fig. 1B-a, e). The slight reductions (4-5%) in the action potential heights were also observed in the test records at 25°C (Fig. 2A-b, c). As a conclusion, the reduction in action potential height by the nicardipine treatment may probably be attributable not to a physiological mechanism to determine its height but to an artificial injury due to the sustaining of an intracellular microelectrode during a contraction.

The result showed the retention of a normal electrical ability over the whole span of the paralyzed fiber excluding other causes such as a local response and/or an inhibition at the end plate level. Our knowledge of “glycerol effect” (Fujino et al., 1961; Howell, 1969) could also exclude the possibility that the paralysis might be related to a T disruption or detubulation, because nicardipine concentrations used were expected not to induce the osmotic shock. Mainly, this paralysis is associated with an interception of the process following a propagated action potential at T membrane.

Inhibition of potassium contracture

The second consideration concerns a contractility in the paralyzed fibers responding to an intense and/or long-lasting depolarization of plasmalemma. The experimental result will lead us to a quantitative understanding for the paralysis depth or inhibitory potency of nicardipine. In order to test this, we used an artificial forced depolarization induced with the depolarization medium containing 190 mEq/l potassium ions. This medium was expected to cause the inside potential about +5 mV and the maximum potassium contracture in a given fiber (Hodgkin and Horowicz, 1959, 1960).

The most important problem of the potassium contracture inducement is the case of a medium exchanging speed. In this study, we adopted a manual-handling flush with Pasteur pipettes. The exchange of a volume of about 1 ml was controlled so as to be finished within a few s. The applicability of this method would be legitimately accepted for the following reasons. The whole length of fibers used was
short and a duration of the full activation or plateau of potassium contractures was long at low temperature (Caputo, 1972b).

Another important problem will be the retention of a normal membrane polarization prior to depolarization inducement. As described already, fortunately it is unlikely that nicardipine treatment itself may injure the retention mechanism. However, other factors such as an injury owing to intense depolarizations evoked repeatedly must be considered. Accordingly, we measured resting membrane potentials preceding the medium exchanging. The measurement procedure was finished within as short a period as possible in order to exclude a development of secondary unfavourable events owing to a microelectrode impalement.

The result is shown in Figs. 3 and 4. All data was obtained from one fiber at 6°C. The fiber was isolated from the frog stocked at room temperature. During repeated stimulations, the depolarization medium was applied after the pretreatment with the choline medium for 1-3 min. Resting potentials were measured twice before and during the respective pretreatments.

As seen in Fig. 4, no remarkable change in resting membrane potentials in normal medium was recognized until 8 h after the removal of nicardipine. Their mean ± S.E. was -90 ± 2 mV (n = 8). Similarly, resting membrane potentials in the choline medium also remained nearly constant, although they appeared as a whole somewhat under those in the normal medium. Their mean ± S.E. was -86 ± 1 mV.
Records a and b in Fig. 3 are of control potassium contractures before the nicardipine treatment. The two contractures with similar size and shape lasted about 10 s in half peak width. This observation at low temperature fundamentally coincided with the work by CAPUTO (1972a).

Records c–i in Fig. 3 are of test potassium contractures recorded after the treatment with 30 μM nicardipine for 14 min. The paralyzed fiber was totally insensitive to the depolarization medium for approximately 1.5 h after the removal of nicardipine (Fig. 3c, d). Before long, the fiber abruptly responded to the depolarization medium with small size and width (Fig. 3e). This inhibition manner was apparently attributable to an acceleration of the relaxation phase. Thereafter, the contracture heights increased gradually depending on time and reached 78% of the control height at 8 h (Fig. 3f–i). The recovery curve connecting the respective contracture peaks appeared roughly exponential with a time-constant of 4.6 h (Fig. 4). The recovery course of twitches appeared with the further slow time-constant of 35 h (Fig. 4).

These observations show that the inhibitory potency of nicardipine is extremely strong compared with known decouplers such as Mn^{2+} (CHIARANDINI and
Retention of s.r. Ca release ability in paralyzed fiber

The last consideration in the paralyzation concerns normalities of s.r. Ca\(^{2+}\) content, its release ability and the ability to develop tension. To verify these normalities, caffeine contracture seems to be most beneficial (WEBER and HERZ, 1968) although several points at issue arise with respect to its generation mechanism (ENDO, 1975). Stimulation with caffeine should be used as little as possible. A threshold concentration in our fibers was around 2 mM in the depolarization medium and agreed with the work by LÜTTGAU and OETLIKER (1968). Here, we adopted 3 mM caffeine.

The result is shown in Fig. 5. Data of action potential, twitch response, and potassium contracture are also shown in order to elucidate both electrical normality and inhibition degree. All data was obtained from one fiber at 6°C. The first group of the records is of control. Caffeine contracture (C) reached a peak at approximately 10 s from its start and lasted at nearly a constant tension level in the caffeine medium. The peak height was 66% of potassium contracture (K) height, which was smaller than that at the high concentration (refer to Fig. 6).

Fig. 5. Effect of nicardipine on caffeine contracture. During repetitive stimulations, 20 µM nicardipine was applied for 30 min duration indicated with white underline. The medium containing 3 mM caffeine was applied for a duration indicated with black underlines: C, control before nicardipine treatment; C', test after the treatment. In order to evaluate nicardipine influence, action potentials (a, a'), twitches (t\(_1\), t\(_2\), t\(_1\)'), and potassium contractures (K, K') are also shown for both controls and tests (dashed characters). Their measurement procedures were similar to those shown already. Resting potential before action potential start was \(-102\) mV in control and \(-108\) mV in test. Fiber, S947.
The caffeine medium was replaced with the normal medium after the immersion for 30 s. The sustained caffeine contracture disappeared immediately. Twitches recovered within 2 min after this replacement (data not shown). These heights were, though, 50% of the control (Fig. 5, $t_1$), which slowly increased and attained a constant level of 58% at 53 min ($t_2$). The fiber contractility at this stage seemed to recover fully, because other fibers treated similarly showed a normal potassium contracture. A source of this incompleteness of twitch tension recovery was unclear but was perhaps due to damage of a certain extent with the long-lasting caffeine contracture. A similar interpretation is also described in the paper by LÜTTGAU and OETLIKER (1968).

The fiber characterized above was treated with 20 μM nicardipine for 30 min. The second group of the records in Fig. 5 was of the test which was obtained at about 16 min after the removal of nicardipine. Response behaviours of the test action potential ($a'$) and the twitch tension ($t'$) were similar to those already described. The inhibition degree was about a fourth when converting to a ratio of potassium contracture heights ($K'/K$), whereas the test caffeine contracture ($C'$) was completely identical to that of the control with respect to its rise and height. The result showed that an ability of s.r. Ca release by caffeine stimulation and the following steps ending in tension development in the paralyzed fiber retained 100% normality.

As described, the paralyzation with nicardipine is properly attributed to an interception of the restricted E-C coupling process. In a strict sense, this process is
preceded by a propagated action potential or depolarization at T membrane and followed by s.r. Ca release. Nicardipine can thus be called a specific decoupler.

Quantitative aspect in dose and temperature

Figure 6 shows quantitative aspects of the inhibition due to nicardipine. In these experiments, most fibers treated with nicardipine under different conditions also retained normal electrical and Ca release abilities: Mean ± S.E. of their resting membrane potential, action potential height, and caffeine (15 mM) contracture height versus control potassium contracture height were $-102\pm2\text{mV}$ ($n=10$), $142\pm3\text{mV}$ ($n=10$), and $87\pm5\%$ ($n=6$), respectively.

Figure 6A shows the relation between the concentrations of nicardipine used and the inhibitory responses. As seen in the figure, two curves connecting respective data points consist of an opposite sigmoidal shape. Concentrations of both threshold and half maximum inhibition were 1 and $1.5\mu\text{M}$ for twitch and similarly 5 and $12\mu\text{M}$ for potassium contracture. Figure 6B shows the relation between the medium temperatures and the inhibitory responses. Both curves consist of a sigmoidal shape with half maximum inhibition at $15^\circ\text{C}$ for twitch and at $12^\circ\text{C}$ for potassium contracture.

DISCUSSION

The present experimental results are summarized. Namely, nicardipine has a strongly marked capacity as a so-called specific decoupler under the condition at low temperature according to our proper definition for E-C coupling. This summary fundamentally agrees to the case of D-600 except that another condition of plasmalemmal depolarization is indispensable to the paralysis establishment by D-600 (EISENBERG et al., 1983). Hereafter, we interpret its action mechanism and propose a working hypothetical model from the standpoint of an E-C coupling concept.

Macromolecular image in paralyzation. The first interpretation concerns a macromolecular mechanism of the nicardipine paralysis. It is safe to assume that the nicardipine treatment did not give a permanent lesion to the architecture responsible for E-C coupling at an electron microscope (EM) level like the D-600 case (EISENBERG et al., 1983). Its target therefore is an elaborate moiety which is unrelated to EM phenomenon and located on and/or in key substance(s) such as protein(s) responsible for E-C coupling.

Since nicardipine is called hydrotropic (IWANAMI et al., 1979), it may penetrate into a hydrophilic domain of the protein “active site” and physicochemically modify its function causing an interference of the protein normal conformation change like the allosteric enzyme-effector interaction case. From the briefly interpreted action mechanism of this drug, we can also attain the widely accepted idea that an E-C coupling concept may have a dynamic aspect related to macromolecule protein behaviour.
Target protein and its subsidiary role in E-C coupling. The second interpretation concerns a nicardipine receptor or target protein and its function. Several biochemical works using skeletal muscle membrane preparations are reported for extraction and purification of protein(s) bound with dihydropyridines (Fosset et al., 1983; Kazazoglou et al., 1983; Borsotto et al., 1984a, b). It is common knowledge that dihydropyridines bind with voltage-dependent Ca channel protein(s) located at T membrane. Its molecular weight, however, seems to disperse. For example, the molecular weight in the case of derivative PN200-110 are 32, 33, and 142 kD (Borsotto et al., 1984a). This dispersion may perhaps account for the protein fragmentation in the course of purification. On the other hand, there is a controversial report that most binding sites of PN200-110 are not functional Ca channels (Almers et al., 1985). These findings suggest that PN200-110 binding sites may be relatively non-specific and that a single protein made up from a few component polypeptides among the fragments may operate as a physiological Ca channel. Tentatively assuming a similar protein in the case of nicardipine, we treat its target protein as a single and functional Ca channel in the following descriptions.

An exact location of Ca channel protein remains unclear. In freeze-fracture EM observation, the luminal leaflet of T membrane is smooth, whereas the cytoplasmic leaflet in the junctional region contains distinctive particles like macromolecule proteins (Franzini-Armstrong and Nunzi, 1983). Therefore, some particles among them may correspond with the Ca channel protein.

Nicardipine paralysis could be also observed when fibers were cooled after nicardipine was completely washed out (Sato and Fujino, in preparation). This finding suggests that the complex formation between nicardipine and its target protein may be temperature-independent and will be compatible with the above protein image. In other words, this target protein of the functional Ca channel is a subsidiary not responsible for E-C coupling since the chemical modification by nicardipine at normal temperature did not produce the decoupling.

Cooperation of physicochemical factors in paralysis. The last interpretation concerns a temperature effect. As a matter of course, the paralysis was established by cooperative effects of both the physical factor of low temperature and the chemical factor of nicardipine treatment. However, a macromolecular mechanism of this key temperature effect is not clear.

It is well known that membranous phenomena during excitation are closely associated with ambient temperature. For example, gradual changes in temperature produce an abrupt transition of the membrane potential in the squid giant axon perfused internally with dilute salt solutions (Inoue et al., 1973). On the other hand, an abrupt cooling produces s.r. Ca release in the caffeinized skeletal muscle, namely rapid cooling contracture (Sakai, 1972; Konishi et al., 1985). Both phenomena, though apparently being widely different, seem to arise from a common root in a swift conformation change of respective membranous macromolecule components, namely channel proteins, responding to temperature.

As described above, the protein conformation change will be essentially
temperature-dependent. This idea is also supported by the following finding in this study: width of action potential at low temperature was very large ($Q_{10} = 5$). This finding may be attributed to the change in delayed rectification (NAKAJIMA et al., 1962), namely slow activation of K channel associated with its lingering conformation change. Similarly, the behaviour of the nicardipine target protein will also be temperature-dependent. In this case, its conformation change seems to be unrelated to membrane excitation. Accordingly, nicardipine-protein complex formed in the manner of temperature-independence may take a "deformation" state only on cooling.

**Two component-three state model.** Here, we propose a tentative model based on the above interpretations (Fig. 7). This model fundamentally resembles a "mechanical" model based on membrane charge movement experiments (RAKOWSKI, 1981). The physical image will be also formed from a molecular model for voltage-dependent channel formation in the planar lipid bilayer membrane (LATORRE and ALVAREZ, 1981). As previously mentioned, the protein receiving nicardipine seems not to play a dominant part but to be subsidiary in E-C coupling. Therefore, we postulate a separate component protein competent for E-C coupling, which can respond to plasmalemmal depolarization with a change in its conformation and is related to the opening of s.r. Ca channel. This protein may be identical to that receiving a sulfhydryl group reagent (FUJINO et al., 1986). The following interaction between these two components will be also supported by the finding that paralysis by a thiol-disulfide interchanging agent cystamine was intensely promoted by the nicardipine pretreatment (FUJINO et al., 1986).

As seen in Fig. 7, the model consists of two components which take three distinct states (two component-three state model). The component A competent for
E-C coupling changes from "resting" state I to "excitation" state II responding to T membrane depolarization (DEP) and returns to the original by the repolarization (REP), whereas the subsidiary component B binds with nicardipine to shift from "normal" state I to "deformation" state III on cooling. This deformation restricts the conformation change of the component A. As a result, the component A is unable to behave normally in E-C coupling.

This model is still primitive. The comparison with the examination of the several models for E-C coupling proposed hitherto (OETLIKER, 1982; CAILLÉ et al., 1985) must be carried out. Further studies will also be required for the interpretation of the detailed action mechanism of nicardipine, in particular, temperature-dependence in the binding with the target protein.

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