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Nisoldipine-induced relaxation in intact and skinned smooth muscles of rabbit coronary arteries

T. Itoh, Y. Kanmura, H. Kuriyama & H. Suzuki

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 In smooth muscles of the rabbit coronary artery, nisoldipine inhibited the phasic and tonic responses of the contraction induced by 128 mM K (the IC_{50} values were 4×10^{-8} M and 1×10^{-13} M, respectively). This agent also inhibited the tonic response of the acetylcholine (ACh) (10^{-5} M)-induced contraction (the IC_{50} value was 3×10^{-10} M), but only slightly inhibited the phasic response (in 10^{-7} M, 0.86 times the control).

2 Nisoldipine ($< 10^{-7}$ M) had no effect on the K-induced depolarization of the membrane at any given concentration. This drug (5×10^{-8} M) did inhibit the oscillatory potential changes and spike potential evoked on the ACh-induced slow depolarization.

3 After depletion of stored Ca from the polarized muscles (5.9 mM K), muscle cells accumulated Ca by application of 2.6 mM Ca without generation of contraction, i.e. a subsequently applied 20 mM caffeine produced the contraction in Ca-free solution. Nisoldipine ($< 10^{-7}$ M) had little effect on this accumulation of Ca.

4 The rate of rise and time to reach the maximum amplitude of the 128 mM K- or ACh-induced contraction (in 2.6 mM Ca) depended on the amount of stored Ca in cells. Nisoldipine (10^{-8} M) consistently inhibited the Ca-induced contraction evoked in depolarized muscles (128 mM K), regardless of the amount of stored Ca. However, this agent (10^{-8} M) did not inhibit the Ca release from storage sites evoked by activation of the muscarinic receptor.

5 After prolonged superfusion (over 120 min) with Na- and Ca-free solution (guanethidine and atropine were present), application of 2.6 mM Ca produced contraction which was inhibited by 10^{-8} M nisoldipine, while the depolarization induced by application of these solutions was not inhibited by nisoldipine.

6 In saponin-skinned muscles, nisoldipine had no effect on the contractile proteins, as estimated from the pCa-tension relationship, or on the Ca accumulation into and Ca release from the Ca storage sites, as estimated from the caffeine-induced contraction.

7 It is concluded that nisoldipine possesses a selective inhibitory action on voltage-dependent Ca influx, when the Ca channel is activated by depolarization.

Introduction

Intracellular (myoplasmic) free Ca plays an essential role in contraction or relaxation of vascular smooth muscle tissue. Increase in free Ca (to concentrations over 10^{-7} M) requires influx of this ion either through activation of the voltage-dependent Ca channel (electrical or K depolarization) or by receptor-activated Ca influx and also by release of Ca stored in the cell (Bolton, 1979; Johnsson & Somlyo, 1980; Jones, 1981; Kuriyama *et al.*, 1982).

Ca antagonists (Ca channel blockers) are drugs which inhibit the influx of Ca at the sarcolemma. These Ca antagonists are classified into three subgroups, on the basis of their chemical structure and include verapamil (verapamil or D-600), diltiazem (diltiazem) and the nifedipine group (nifedipine, nimodipine, nitrendipine, nisoldipine or nicardipine). Since the chemical structure of individual drugs differs, the action of these agents on cardiac and vascular muscles differs with regard to their potency in inhibiting Ca influx (Fleckenstein, 1977, 1983; Cauvin *et al.*, 1983).

The nifedipine group possesses the most potent vasodilator action, compared with other groups (Grun & Fleckenstein, 1972; Jatene & Lichtlen,

1976; Puech & Krebs, 1980; Fleckenstein, 1983). Makita *et al.* (1983) compared the inhibitory actions of individual agents of the nifedipine group on smooth muscle cells of the rabbit mesenteric artery, and concluded that they inhibited the Ca-spike as well as contractions caused by 128 mM K and high concentrations of noradrenaline (NA). However, these agents appeared to have minor effects on the adrenoceptor-activated Ca increases in the myoplasm which occurred in the absence of depolarization by application of low concentrations of NA. They did not inhibit the NA or caffeine-induced contraction in Ca-free EGTA containing solution. Golenhofen (1981) found that nifedipine inhibited the contraction induced by NA in the portal vein, ear and coronary arteries and the aorta, but that the potency of the inhibitory action differed with the tissue. The contribution of nifedipine-sensitive and nifedipine-resistant Ca influx for generation of the contraction varied with different tissues.

Among the nifedipine group, nisoldipine possesses the most potent inhibitory action on the contraction evoked in the rabbit mesenteric artery (Makita *et al.*, 1983); hence, nisoldipine is the most potent Ca antagonist available.

In the rabbit coronary artery, the acetylcholine (ACh)-induced contraction showed a similar amplitude to that of the K-induced contraction; therefore, we have examined the mechanism of inhibition of nisoldipine on the contraction evoked by high concentrations of K, ACh or caffeine in intact and chemically skinned muscles prepared from the rabbit coronary artery.

Methods

Albino rabbits of either sex (1.8–2.2 kg) were given sodium pentobarbitone (40 mg kg^{-1} i.v.) and exsanguinated. The coronary artery was immediately placed in a dissecting chamber filled with Krebs solution. Circular strips were prepared from the left anterior descending coronary artery (diameter; 1.0 mm).

Solution

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9 Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.4 and glucose, 11.5. High-$[K]_o$ solution was prepared by replacing NaCl, isosmotically, and Na-free solution was prepared by replacing Na either with choline chloride or Tris-chloride. The pH of the Na-free (choline) solution was adjusted to 7.2 with Tris buffer. In Na-free (choline) solution, atropine 10^{-6} M was present throughout the experiments on intact muscles.

Recording of electrical activity

In the microelectrode experiments, the dissected coronary artery (1.0 mm in width and 10–15 mm in length) was mounted in an organ bath with a capacity of about 2 ml and Krebs solution (34–35°C) was superfused at a flow rate of about 3 ml min^{-1}. The electrical activity elicited by ACh on individual regions of the coronary artery differed and, therefore, the precise region of the tissue is described in the Results.

For stimulation of the muscle, the partition stimulating method was used (Abe & Tomita, 1968). A 1–2 s current pulse was applied to the long axis of the tissue. Glass microelectrodes (Hilgenberg Glass, Frankfurt) filled with 3 M KCl and with a tip resistance of 40–80 MΩ were used to impale single cells to record the electrical activities of the muscle membrane (Nihon Kohden Recticorder, RJG(4024)).

Recording of mechanical activity

Mechanical responses were measured by attaching a circular strip excised from left anterior descending coronary artery (0.05–0.08 mm in width and 0.4 mm in length) to a strain gauge (U-gauge, Shinko Seiki). The tissue was superfused in an organ bath (0.9 ml) filled with Krebs solution. Solutions containing drugs or with modified ionic concentrations were added to the bath while the solution already present was aspirated by a water pump; this allowed the test solutions to be applied in only a few seconds. However, this procedure was accompanied by large artifacts due to sudden changes in the level of the solution in the organ bath. Artifacts were ruled out as described previously (Itoh *et al.*, 1981).

Mechanical responses of the coronary artery evoked by excess concentrations of K were measured after equilibration with tetrodotoxin (TTX) 10^{-7} M and with guanethidine 10^{-6} M.

Skinned muscle preparations were obtained by using saponin, according to the method described by Itoh *et al.* (1981), i.e. after a K-induced contraction (128 mM) of the intact muscle had been recorded, the bathing solution was replaced with a relaxing solution containing 130 mM K methansulphonic acid (Ms), 20 mM Tris-maleate, 5 mM $MgMs_2$, 5 mM ATP (10 mM Na as Na_2 ATP) and 4 mM EGTA at pH 6.8. The preparation was left for 20 min in the relaxing solution containing 25 μg ml^{-1} saponin (ICN) and was washed again with the same solution and left until the tension level became constant at about zero level. Immediately before application of a Ca containing solution, the preparation was superfused with the relaxing solution. To investigate the effects of nisoldipine on calmodulin, including contractile proteins, the free Ca-tension relation curves (pCa-

tension relationship) in the presence or absence of nisoldipine were observed. Various Ca concentrations were prepared by adding appropriate amounts of $CaCl_2$ to EGTA. The apparent binding constant of EGTA for Ca was considered to be $10^6\ M^{-1}$ at pH 6.8 at 25°C (Itoh *et al.*, 1981). The pH of the relaxing and various Ca solutions was kept at 6.8 by adding KOH instead of KCl, isotonically.

Chemicals used in the experiments were nisoldipine (Bayer), caffeine (Wako), saponin (ICN), acetylcholine Cl and tetrodotoxin (TTX; Sigma), guanethidine and tetraethylammonium Cl (TEA; Tokyo Kasei), and ethyleneglycol-*bis* (β-aminoethylether)-N, N'-tetraacetic acid (EGTA; Dozin). These drug solutions were freshly prepared before each experiment. Nisoldipine (3×10^{-4} M) was originally dissolved in 96% ethanol and polyethylene glycol (150 mg ml^{-1}); therefore, to avoid the related effects, nisoldipine was used in a concentration below 3×10^{-7} M (the concentration of ethanol was below 0.1%). Physiological solution was used for dilution to the proper concentrations, and the pH was adjusted to 7.2. To prevent inactivation of nisoldipine by exposure to light, the experiments were carried out under a sodium lamp.

Results

Effects of nisoldipine on the membrane activity and the phasic and tonic responses of the K- or ACh-induced contraction

Resting membrane potentials of the rabbit coronary artery ranged between −50 and −60 mV (mean value, -53.7 ± 3.1 mV, $n = 37$). In Krebs solution, the cells were electrically quiescent and outward current pulses did not evoke a spike. TEA (1 mM)

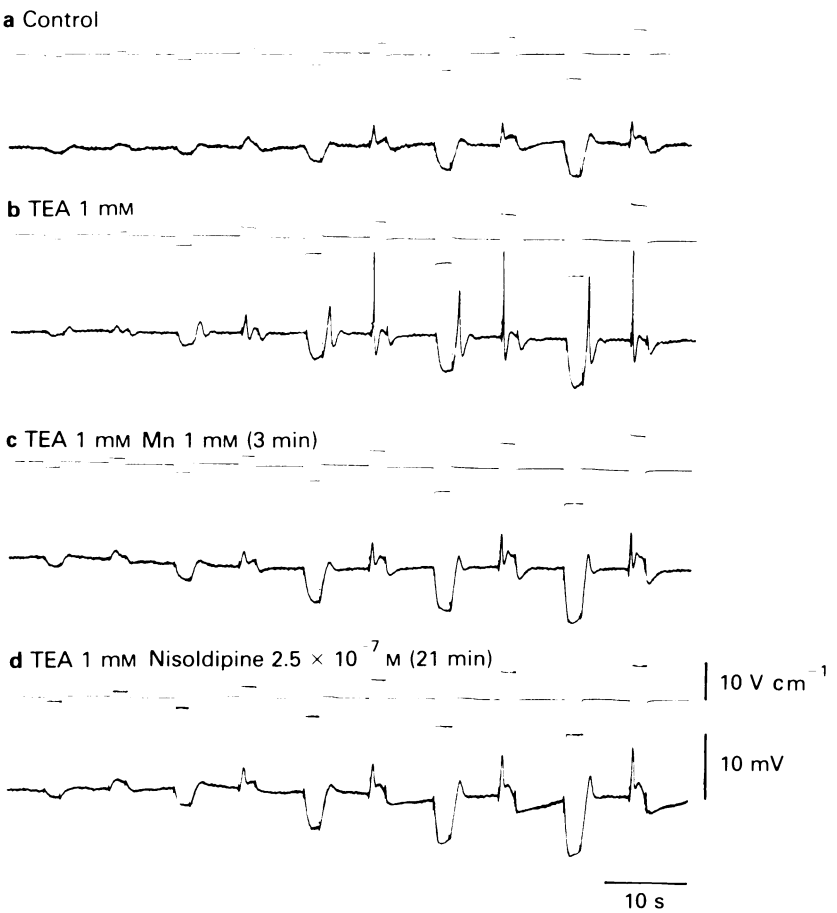

Figure 1 Effects of $MnCl_2$ 1 mM or nisoldipine 2.5×10^{-7} M on the spike evoked by outward current pulses in the presence of tetraethylammonium (TEA) 1 mM. To evoke electrotonic potential and spike, inward and outward current pulses of 2 s duration were applied at 0.1 mm distance from the stimulating electrode.

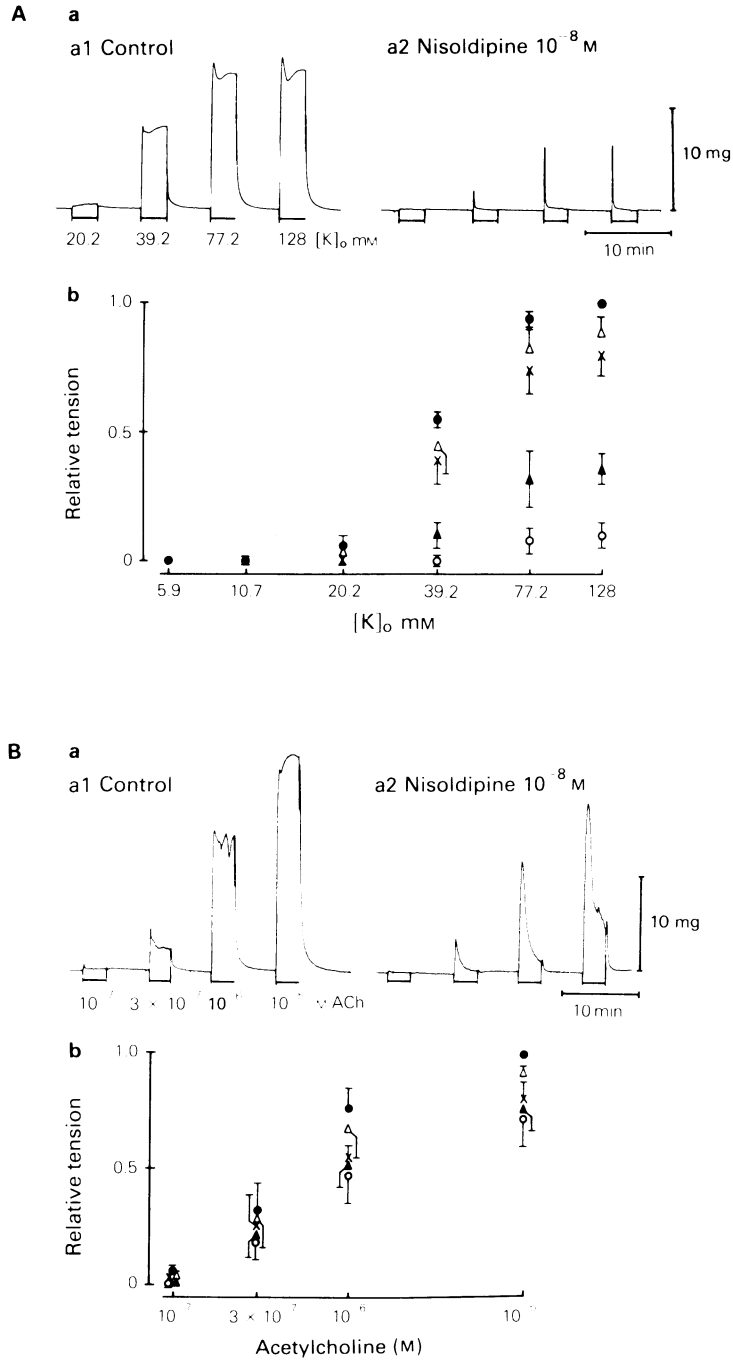

Figure 2 (A) Effects of nisoldipine on the K-induced contraction. Various concentrations of K were applied for 5 min with application of nisoldipine 10^{-8} M in (a2) (a1; control, a2; with application of nisoldipine); (b) effects of nisoldipine on phasic responses of the K-induced depolarization. The amplitude of the phasic response of the 128 mM K-induced contraction was taken as 1.0. $n = 3-6$ and vertical bars indicate $\pm$ s.d. Control (●), nisoldipine 10^{-10} M (△), 10^{-9} M (×), 10^{-8} M (▲) and 10^{-7} M (○). (B) Effects of nisoldipine on the acetylcholine-induced contraction. Explanations of figure and symbols are the same as in A. $n = 4-6$.

depolarized the membrane and evoked spikes by outward current and anodal break pulses in the presence of TTX (3×10^{-7} M) and guanethidine (10^{-6} M). Figure 1 shows the effects of nisoldipine on the action potential evoked by outward current pulses (3 s pulse duration) in the presence or absence of 1 mM TEA. The spike evoked in the presence of 1 mM TEA was blocked by 1 mM $MnCl_2$ or 2.5×10^{-7} M nisoldipine.

Increased concentrations of $[K]_o$ depolarized the membrane. Application of 10^{-7} M nisoldipine did not modify the membrane potential, at any given concentration of K (in 5.9 mM K, control -53.7 ± 3.1 mV, $n = 37$; 10^{-7} M nisoldipine, -53.2 ± 3.2 mV, $n = 17$; in 30.1 mM K, control -32.7 ± 1.5 mV $n = 15$; 10^{-7} M nisoldipine, -33.7 ± 2.8 mV, $n = 16$, and in 90.7 mM K, control -9.8 ± 1.6 mV, $n = 15$, 10^{-7} M nisoldipine, -10.3 ± 1.4 mV, $n = 14$).

Figure 2A shows the effects of various concentrations of nisoldipine (10^{-10}M–10^{-7}M) on the K-induced contraction (10.2 mM–128 mM). The amplitude of the phasic response of contraction evoked by 128 mM K was taken as 1.0 (Figure 2 Ab). The minimum concentration of K required to produce the contraction was 20.2 mM at a membrane potential of -36 ± 1.8 mV, $n = 15$. Application of 10^{-8}M nisoldipine (10 min before and during application of excess concentrations of K), partially inhibited the phasic response and completely inhibited the tonic response evoked by any given concentration of K. As a consequence, a transient sharp contraction was evoked in the presence of nisoldipine (Figure 2 Aal–a2). Figure 2B shows the effects of nisoldipine on the ACh-induced contraction. The contraction could be evoked by applications of ACh in concentrations over 3×10^{-7}M. In Figure 2Bb, the contraction evoked by 10^{-5}M ACh was taken as 1.0. Nisoldipine (10^{-8}M) consistently inhibited the tonic response but only slightly inhibited the phasic response of the ACh-induced contraction. The amplitude of tonic response was measured 5 min after application of ACh (Figure 2 Bb). Figure 3 shows the effects of various concentrations of nisoldipine on the phasic and tonic responses of the 128 mM K- or 10^{-5}M ACh-induced contraction. The IC_{50} values calculated for the action of nisoldipine on the phasic and tonic responses evoked by 128 mM K were 4×10^{-8}M and 1.1×10^{-13}M nisoldipine, respectively, and the value for the tonic response of the ACh-induced contraction was 3×10^{-10}M.

ACh generated three different membrane responses from smooth muscles excised from different regions of the rabbit coronary artery, i.e. a depolarization of the membrane with oscillatory potential changes (proximal part of left anterior descending coronary artery), a transient hyperpolarization which was followed by depolarization with oscillatory potential changes (left anterior descending coronary artery) and hyperpolarization alone (right coronary artery).

Figure 4 shows the effects of nisoldipine on the ACh-induced hyperpolarization of the membrane. The amplitude of the ACh-induced hyperpolarization increased in a concentration-dependent way (Figure 4 Aa and B). The minimum concentration of ACh required to produce the hyperpolarization was 10^{-8} M. Application of 10^{-7} M nisoldipine did not modify the hyperpolarization evoked by any given concentration of ACh (Figure 4 Ab) but application of atropine (10^{-6} M) led to cessation of the hyperpolarization (Figure 4 Ac). Figure 4B shows the ef-

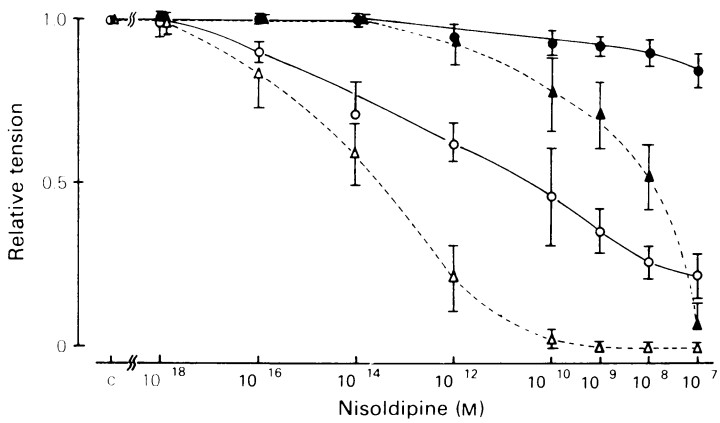

Figure 3 Effects of nisoldipine on the 128 mM K- or 10^{-5} M acetylcholine (ACh)-induced contraction. The amplitude of phasic or tonic responses evoked in the absence of nisoldipine is registered as a relative tension of 1.0. Concentrations of nisoldipine varied from 10^{-18} M to 10^{-7}M. The IC value is measured from 50% reduction in the amplitude of contraction. $n = 4$–7. Vertical bars indicate ± s.d. (▲) $[K]_0$ 128 mM phasic; (△) $[K]_0$ 128 mM tonic; (●) ACh 10^{-5} M phasic; (○) ACh 10^{-5} M tonic.

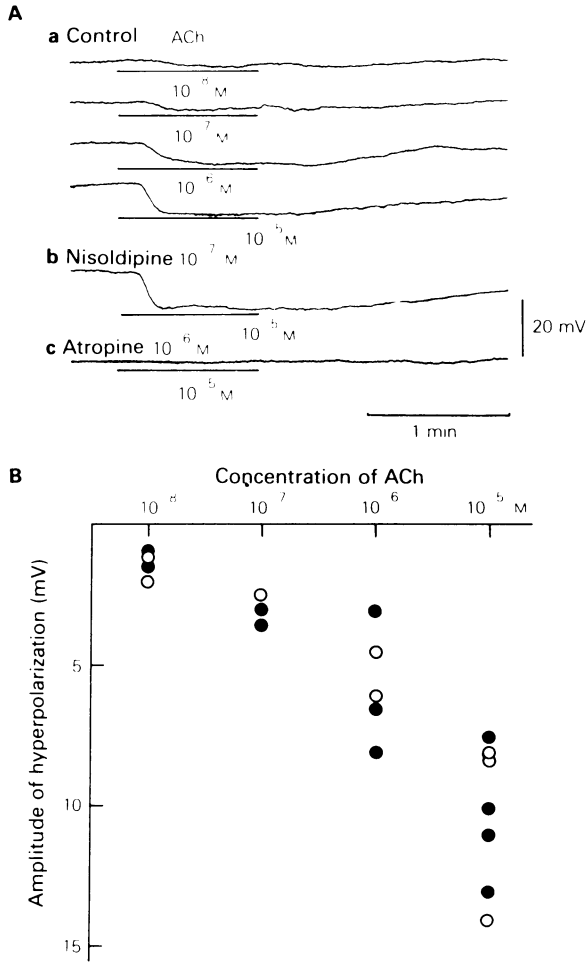

Figure 4 Effects of nisoldipine on the acetylcholine (ACh)-induced hyperpolarization in the smooth muscle cells of the rabbit coronary artery (right decending artery). (A) Actual electrical records. Concentrations of acetylcholine and duration of application are indicated by bars. Nisoldipine 10^{-7} M was present before and during the application (b). ACh (10^{-5} M) was applied after pretreatment (3 min) with atropine (10^{-6} M) (c). (B) Dose-response relationship of the ACh-induced hyperpolarization in the presence (○) or absence (●) of nisoldipine (10^{-7} M). All the points were obtained from the same tissue.

fects of nisoldipine (10^{-7} M) on the amplitude of hyperpolarization produced by application of various concentrations of ACh. Nisoldipine had no effect on the ACh-induced hyperpolarization.

ACh (10^{-8} M) depolarized the membrane of smooth muscles excised from the proximal part of the left anterior descending coronary artery. Increased concentrations of ACh led to further depolarization, and oscillatory potential changes superimposed on the slow depolarization. Figure 5 shows the effects of nisoldipine (5×10^{-8} M) on the ACh-induced depolarization. In this particular cell, ACh produced a transient hyperpolarization which was followed by a large depolarization with oscillatory potential changes. Nisoldipine (10^{-7} M) reduced both the appearance and amplitude of the oscillatory potential changes, but the hyperpolarization and slow depolarization were not affected. In some cells, ACh produced a spike on the oscillatory potential change and this spike was also inhibited by nisoldipine. Even though, ACh either produced hyperpolarization or depolarization of the membrane, this agent consistently produced contraction in circular cut strips.

Effects of nisoldipine on the Ca-, ACh- or caffeine-induced contraction in polarized and depolarized muscles

Figure 6 shows the effects of nisoldipine (10^{-8} M) on the 128 mM K- or 10^{-5} M ACh-induced contraction in various concentrations of Ca (0 mM–7.8 mM). When 128 mM K or 10^{-5} M ACh in Ca-free 2 mM EGTA containing solution was applied to the tissue, ACh but not 128 mM K produced a contraction. The amplitude and shape of contraction evoked by simultaneous applications of 10^{-8} M nisoldipine with 10^{-5} M ACh in Ca-free solution were not affected. When 10^{-8} M nisoldipine, with various concentrations of Ca, were applied simultaneously with 128 mM K or 10^{-5} M ACh, increased concentrations of Ca gradually increased the amplitude of the phasic response of the K-induced contraction, in a concentration-dependent way (0.5 mM–7.8 mM) but the tonic response was not generated. In the case of ACh, increased concentrations of Ca (0.1 mM–7.5 mM) gradually enlarged the amplitude of the tonic response (0.1 mM–7.5 mM Ca) in the presence of 10^{-8} M nisoldipine.

Figure 7A and B show the effects of nisoldipine (10^{-8} M) on Ca accumulation in the cell. To estimate the amount of Ca stored in cells, the following procedures were used: after complete depletion of the stored Ca by repetitive application of caffeine (20 mM) in Ca-free 2 mM EGTA containing solution, various concentrations of Ca were applied for 20 min to accumulate Ca^{2+} in the store site and subsequently 20 mM caffeine was applied following a 2 min superfusion with Ca-free 2 mM EGTA containing solution, for the polarized muscles (5.9 mM K). Application of Ca (up to 7.8 mM) did not produce contraction, but the resulting caffeine-induced contraction was increased in proportion to the applied concentrations of Ca (up to 2.6 mM). Further increased concentra-

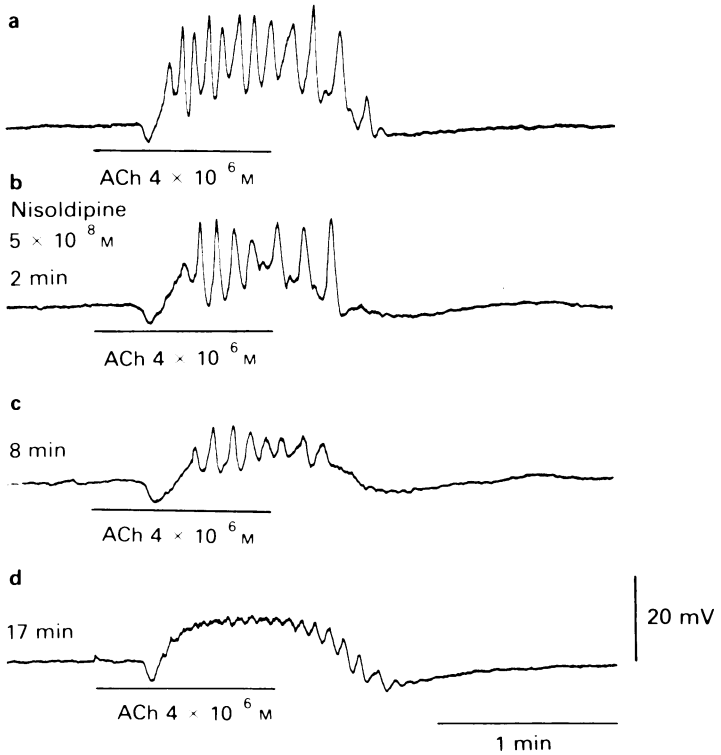

Figure 5 Effects of nisoldipine on the acetylcholine(ACh)-induced electrical activity. Nisoldipine 5×10^{-8} M was applied in the presence of acetylcholine 10^{-6} M. (b–d) The effects 2 min, 8 min and 17 min after application of nisoldipine are shown. The electrical activity in all records was recorded from the same cell.

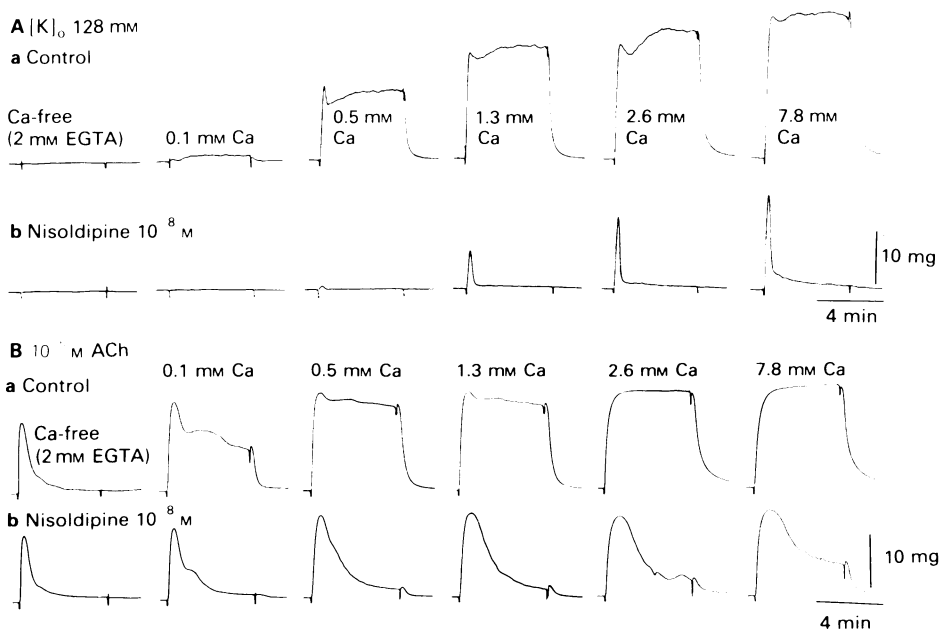

Figure 6 Effects of nisoldipine 10^{-8} M on the 128 mM K-(A) or 10^{-5} M acetylcholine-(B) induced contraction in the presence of various concentrations of Ca. (a) Control; (b) after application of nisoldipine.

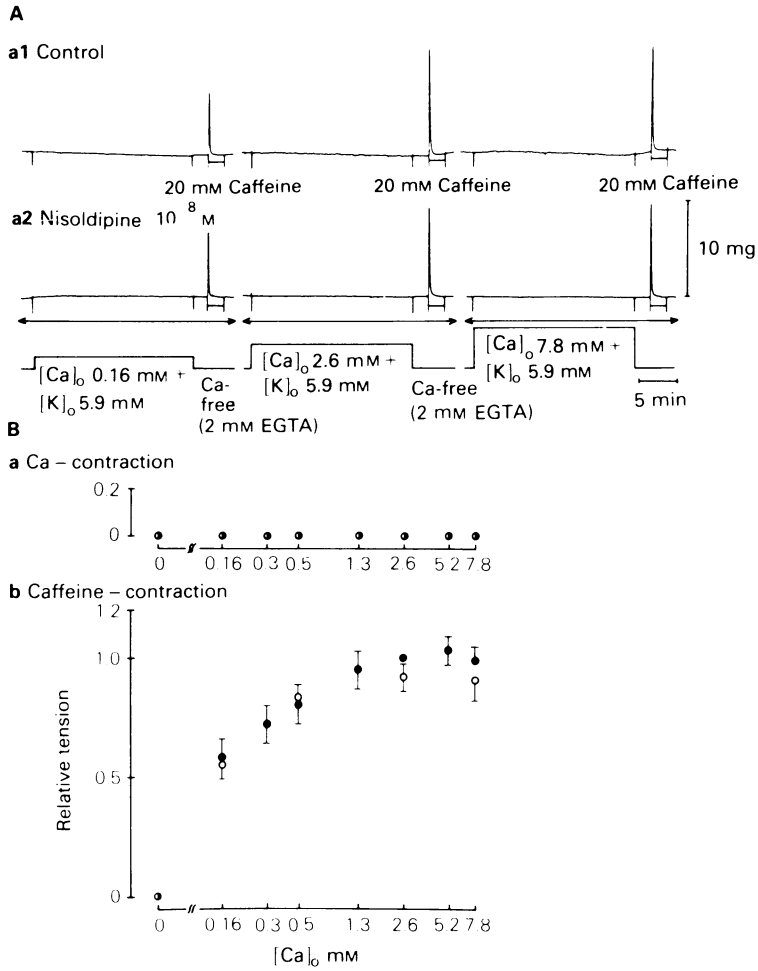

Figure 7 Effects of simultaneous application of Ca and nisoldipine on the subsequently generated contraction evoked in Ca-free 2 mM EGTA containing solution. Various concentrations of Ca were applied for 20 min in polarized muscles (5.9 mM K). (A) Actual records before (a1) and during (a2) application of nisoldipine. Arrows indicate application of 10^{-8} M nisoldipine. (B) Relationship between applied Ca concentration and the Ca-induced contraction (a) or subsequently generated caffeine-induced contraction (b) in Ca-free 2 mM EGTA containing solution in the presence (○) or absence (●) of nisoldipine 3×10^{-9} M. Vertical bars indicate ± s.d. $n = 5-7$. Details are described in the text.

tions of Ca slightly enhanced the amplitude of the caffeine-induced contraction. When 3×10^{-9} M nisoldipine was applied to the tissue throughout the experiments (before and during addition of various concentrations of Ca and subsequently applied Ca-free solution), the resulting caffeine-induced contraction was little affected (B).

Figure 8A shows the effects of nisoldipine (10^{-12} M or 3×10^{-9} M) on the Ca-induced, and the subsequently generated caffeine-induced, contractions following treatment with various concentrations of Ca in depolarized muscles (128 mM K). In this experiment, 2.6 mM Ca was applied for 5 min to accumulate Ca into the store site. The amplitude of the Ca- or caffeine-induced contraction was proportionally increased by raised Ca concentrations. When 10^{-8} M nisoldipine was applied simultaneously with various concentrations of Ca, both the Ca- and caffeine-induced contractions were inhibited at any given concentration of Ca. However, after complete inhibition of the Ca-induced contraction by application of 3×10^{-9} M nisoldipine, the caffeine-induced contraction was still generated but with a reduced amplitude. Figure 8B shows the relationship between

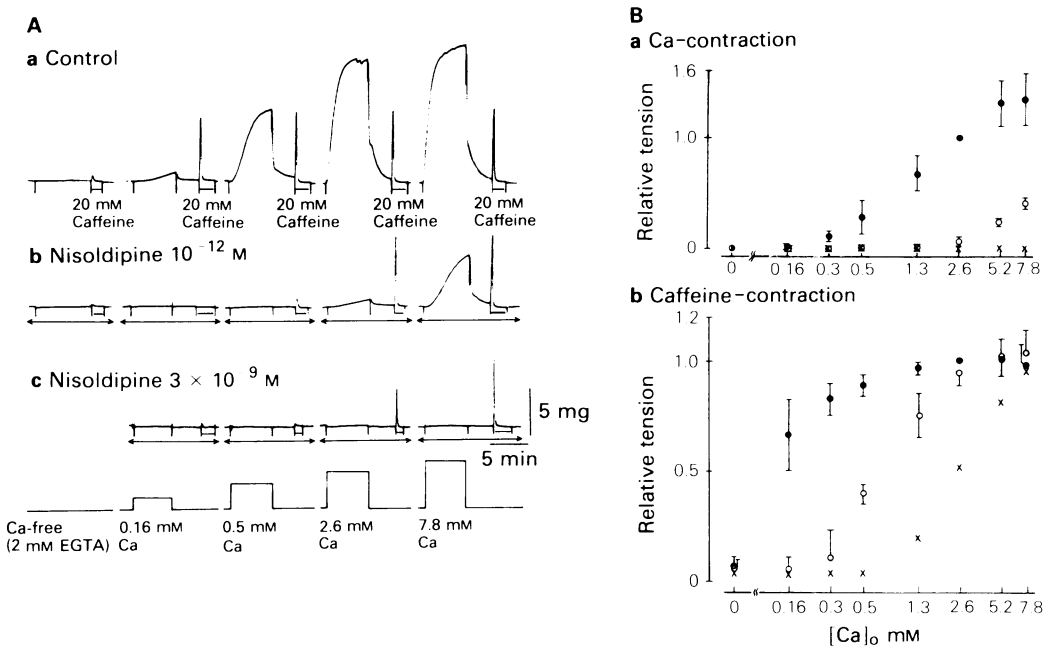

Figure 8 (A) Effects of simultaneous application of various concentrations of Ca and nisoldipine on the Ca-induced and subsequently generated caffeine-induced contractions in 128 mM K containing solution. Various concentrations of Ca (0–7.8 mM) were applied for 5 min. (a) Control; (b) nisoldipine 10^{-12} M; (c) nisoldipine 3×10^{-9} M. Bar with arrows indicate presence of nisoldipine. (B) Effects of nisoldipine on the Ca-(a) and subsequently generated caffeine-(b) induced contractions in the presence of various concentrations of Ca with 128 mM K. (a) Ca-induced contraction; (b) caffeine-induced contraction. Vertical bars indicate ± s.d., $n = 5-7$. In (a) the 2.6 mM Ca-induced contraction was taken as 1.0, and in (b) the caffeine-induced contraction in Ca-free solution following application of 2.6 mM Ca was taken as 1.0. In (a) and (b), (●) control; (○) 10^{-12} M nisoldipine; (×) 3×10^{-9} M nisoldipine.

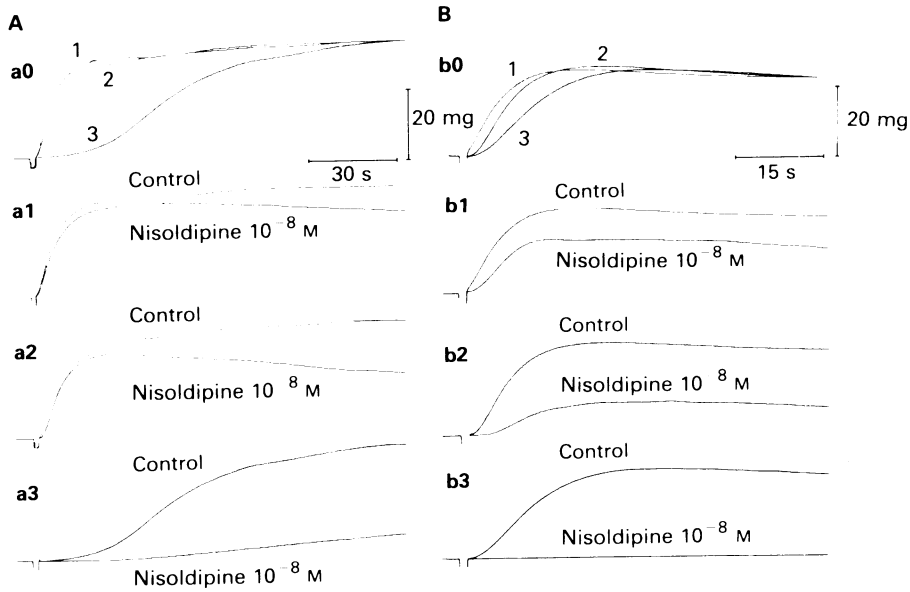

Figure 9 Contributions of stored Ca for generation of 10^{-5} M acetylcholine- or 128 mM K-induced contraction in the presence or absence of nisoldipine. (A) Acetylcholine(ACh)-induced contraction: (1) effects of acetylcholine in Krebs solution, (2) effects of simultaneous application of acetylcholine with 2.6 mM Ca in Ca-free 2 mM EGTA containing solution; (3) effects of simultaneous application of acetylcholine with 2.6 mM Ca after depletion of Ca stored in the cell by repetitive application of 20 mM caffeine in Ca-free solution.
(a0) Actual records of contractions evoked under procedures 1–3 were superimposed. (a1–a3) Effects of Ca with acetylcholine under different conditions of stored Ca (1–3) in the cell in the presence or absence of nisoldipine 10^{-8} M. (B) With the same experimental procedures as (A) but K 128 mM was applied instead of acetylcholine.
(b0) superimposed 128 mM K with 2.6 mM Ca-induced contractions evoked with three different procedures (1–3); (b1–b3) effects of nisoldipine on the contraction evoked by 128 mM K with 2.6 mM Ca.

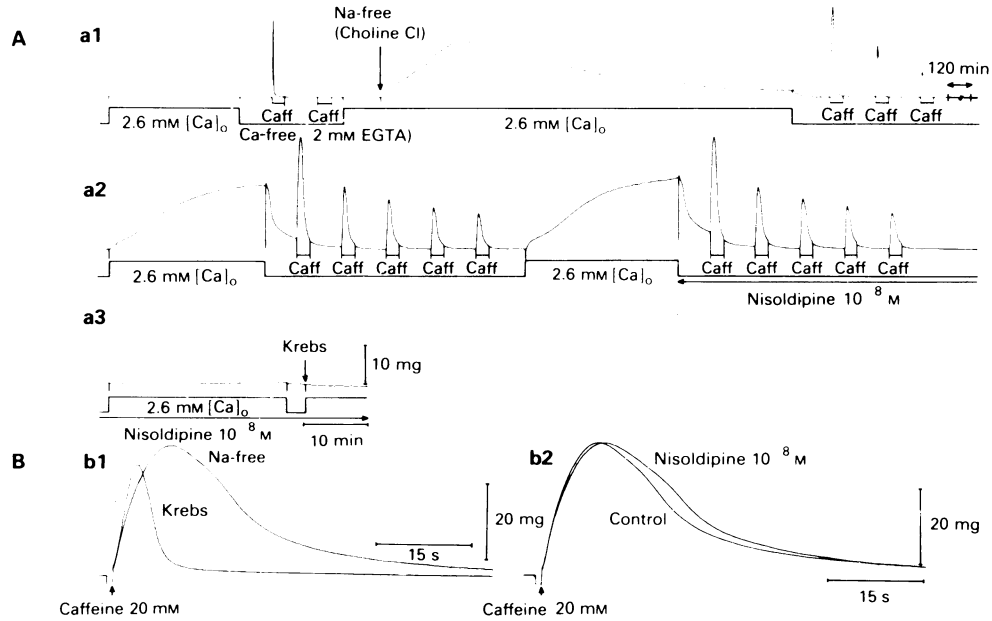

Figure 10 Effects of nisoldipine on the contractions evoked by caffeine and Ca in Na-free solution (choline substituted for Na with atropine 10^{-6} M). (A) (a1–a3) Continuous records. (a1) After depletion of Ca in storage sites by repetitive application of caffeine, Ca 2.6 mM was applied for 20 min and subsequently caffeine 20 mM was applied 2 min after application of Ca-free 2 mM EGTA containing solution. A second application of caffeine (20 mM) did not produce the contraction. The above experiments were done for normalization of the caffeine-induced contraction. When Na was removed from Krebs solution in the presence of Ca (2.6 mM Ca), the contraction was evoked. After reduction in the amplitude of contraction to the resting level (after 60 min), caffeine was applied 3 times in Ca-free 2 mM EGTA containing solution. (a2) After prolonged exposure in Na- and Ca-free solution, application of Ca produced contraction; subsequently repetitively applied caffeine produced large contractions with decay in amplitude. In the latter half of the record, nisoldipine was applied during application of caffeine in Na- and Ca-free solution. (a3) Ca 2.6 mM is applied with nisoldipine in the absence of Na. (B) Effects of nisoldipine on the subsequently generated caffeine-induced contraction in Na- and Ca-free solution. (b1) The caffeine-induced contraction in the presence or absence of Na (Na-free indicates the contraction evoked by application of the first caffeine trial (a2) in Na- and Ca-free solution). (b2) Effects of nisoldipine on the caffeine-induced contraction evoked in Na- and Ca-free solution (in series of a2).

the Ca-induced contractions (a) the subsequently generated caffeine-induced contractions (b) and Ca concentrations, in the presence or absence of 10^{-12} M or 3×10^{-9} M nisoldipine, respectively. After complete depletion of the stored Ca, application of Ca (0.3 mM) with 128 mM K led to contraction. When the amplitude of the 2.6 mM Ca-induced contraction was taken as 1.0, increased concentrations of Ca further increased the amplitude. With application of the solution containing Ca, 128 mM K and 10^{-12} M nisoldipine, the amplitude of the Ca-induced contraction was markedly inhibited and with application of 3×10^{-9} M nisoldipine, the generation of the Ca-induced contraction (up to 7.8 mM Ca) ceased. The caffeine-induced contraction (2×10^{-2} M) could be evoked in Ca-free 2 mM EGTA containing solution when 0.16 mM Ca was previously loaded, (the amplitude was 0.07 times the control; $n = 5$, 2.6 mM Ca treatment was normalized as 1.0), without generation of the Ca-induced contraction. With applications of nisoldipine (10^{-12} M or 3×10^{-9} M) and 128 mM K, the amplitude of the 2.6 mM Ca-induced contraction ceased (Ba), while the resulting caffeine-induced contraction showed almost the maximum amplitude (Bb). Inhibitory actions of nisoldipine appeared to be chiefly on the Ca^{2+}-induced contraction.

Effects of nisoldipine on the Ca-induced Ca release mechanism in relation to the amount of Ca stored in cells

The Ca-induced contraction in 128 mM K had a causal relation to the amount of Ca stored in the cell, i.e. to produce the Ca-induced contraction, a certain amount of stored Ca was required. To determine, whether or not this phenomenon is related to the Ca-induced Ca-release mechanism for generation of the contraction, the contraction was evoked by

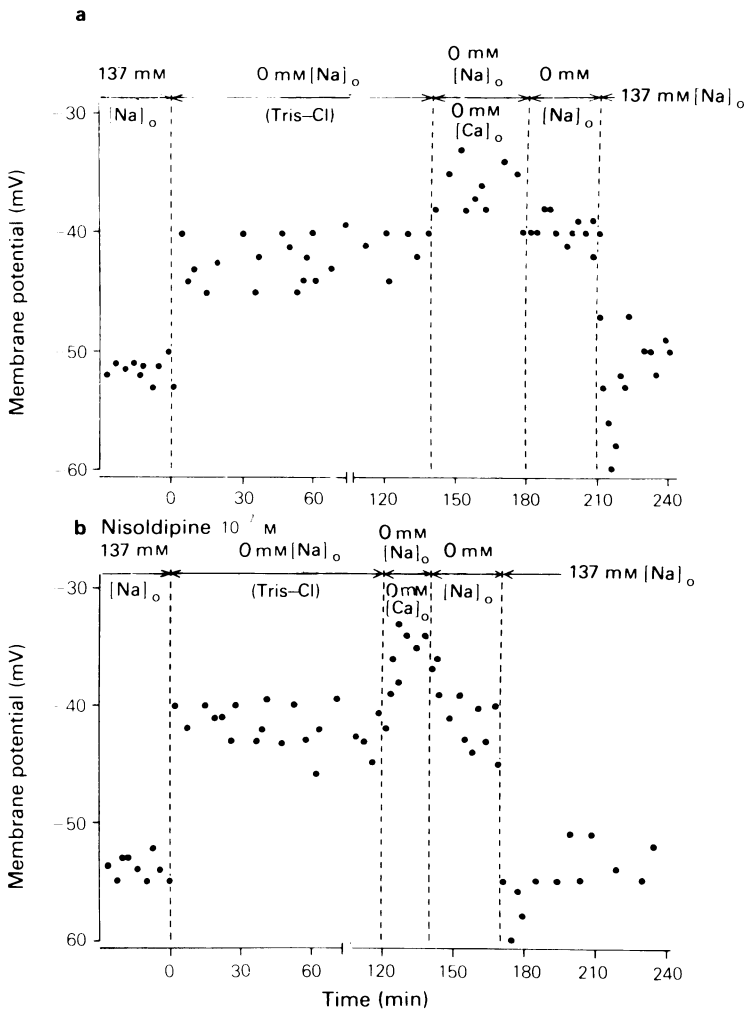

Figure 11 Effects of 10^{-7} M nisoldipine on the membrane potential measured in Na-free (Tris), and Na- and Ca-free solutions. (a) Control; (b) nisoldipine 10^{-7} M was applied throughout the experiment. Dots indicate the membrane potentials obtained by microelectrode impalements. (a) and (b) were recorded from different tissues.

2.6 mM Ca with 128 mM K or 2.6 mM Ca with 10^{-5} M ACh following different conditions of Ca storage in the cell.

In Figure 9Aa0 and Bb0, the contractions evoked in different conditions are superimposed: i.e. in (1) the contraction was evoked by 10^{-5} M ACh (also indicated as control in a1) or 128 mM K (b1 control) in 2.6 mM Ca containing solution: (2) the contraction was evoked by 2.6 mM Ca with ACh (a2 control) or with 128 mM K (b2 control) after 5 min superfusion with Ca-free solution: (3) the contraction was evoked by 2.6 mM Ca with ACh (a3 control) or with 128 mM K (b3 control) after depletion of stored Ca by repetitive application of 20 mM caffeine in Ca-free 2 mM EGTA containing solution. The results obtained can be summarized as follows; (i), the rate of rise of the contraction evoked by ACh or 128 mM K is dependent on the amount of Ca stored in the cell; (ii), the peak amplitude remains the same regardless of the amount of Ca stored, however, the time required to reach the peak amplitude is dependent on the amount of stored Ca.

As shown in Figure 9 a1–3 and b1–3, nisoldipine inhibited the contraction under various conditions of stored Ca. After depletion of the stored Ca, nisoldipine completely blocked generation of the K-induced contraction (b3) but the ACh-induced contraction (a3) which gradually developed had a long

latency and slow rate of rise. Part of the nisoldipine-resistant Ca influx may contribute to the generation of the ACh-induced contraction. If the Ca influx directly activates the contractile protein, the same amount of Ca influx may produce the same rate of rise and the time to reach the peak amplitude of contraction with no relation to the stored Ca. However, the results showed that the contraction was evoked after replenishment of the Ca in the storage site.

Effects of nisoldipine on the caffeine-induced contraction evoked in Ca- and Na-free solution

In various vascular smooth muscle tissues, repetitive generation of the contraction evoked by caffeine or NA persisted longer in the Ca- and Na-free solution, than those evoked in Ca-free (2 mM EGTA containing) solution (Itoh *et al.*, 1983 a,b; Kanmura *et al.*, 1983 a,b). The effects of nisoldipine on the caffeine-induced contraction (2×10^{-2} M) were observed in Ca- and Na-free (choline with 10^{-6} M atropine) solution. As shown in Figure 10, after Ca had been loaded, the subsequently applied 20 mM caffeine produced a contraction, 5 min after application of Ca-free 2 mM EGTA containing solution, but the second application of 20 mM caffeine did not produce contraction. In the presence of 2.6 mM Ca, Na-free solution produced a contraction, and the tissues gradually relaxed to the control level after about 1 h. This contraction was blocked by application of nisoldipine. When the tissues was rinsed with Na- and Ca-free 2 mM EGTA containing solution, the amplitude and duration of the subsequently generated caffeine (20 mM)-induced contraction were enhanced, and repetitive application of caffeine successively produced contractions with a gradual reduction in the amplitude (Figure 10a1). When 2.6 mM Ca was applied after 220 min superfusion in the Ca- and Na-free solution, the tissue produced a contraction and the subsequently generated caffeine-induced contractions were further enlarged (a2). Nisoldipine (10^{-8} M) did not modify the amplitude of the contraction evoked by repetitive application of caffeine (a2). However, the Ca-induced contraction ceased with application of 10^{-8} M nisoldipine (a3). When the shapes of the caffeine-induced contractions evoked after Ca loading in the presence or absence of Na were compared, amplitude and duration of the contraction were both enhanced in the absence of Na (B b1). Nisoldipine did not modify the shape of the resulting caffeine-induced contraction evoked in Na- and Ca-free solution (after 220 min for the control and 225 min in the presence of nisoldipine in B b2).

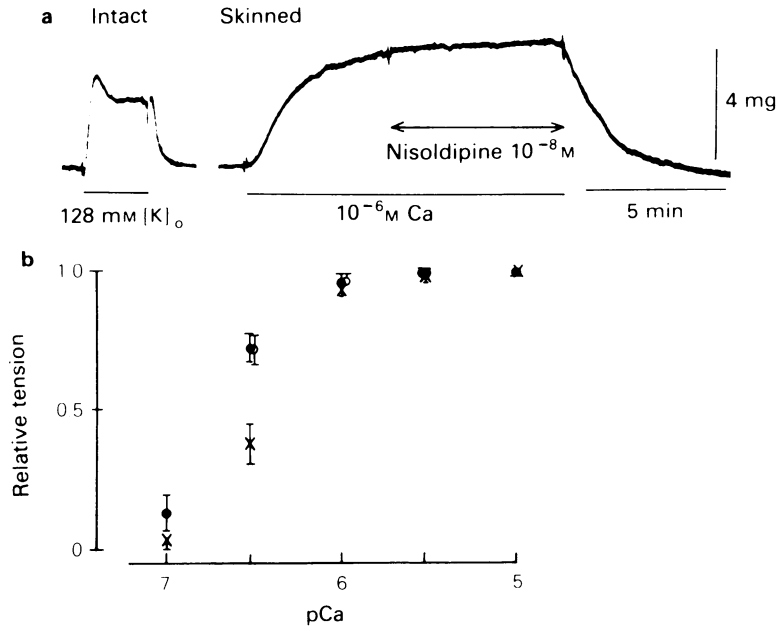

Figure 12 Effects of nisoldipine on the Ca-induced contraction in skinned muscles. (a) Intact: 128 mM K is applied to the tissue before skinning the tissue; Skinned: after skinning the tissue with saponin (see Methods), 10^{-6} M Ca was applied. Nisoldipine was applied after the Ca-induced contraction reached a steady amplitude. (b) The effects of nisoldipine on the free Ca (pCa)-tension relationship observed in skinned muscles. Coronary artery: control (●); nisoldipine 10^{-8} M (○); mesenteric artery control (×). Vertical bars indicate ± s.d., $n = 3-5$.

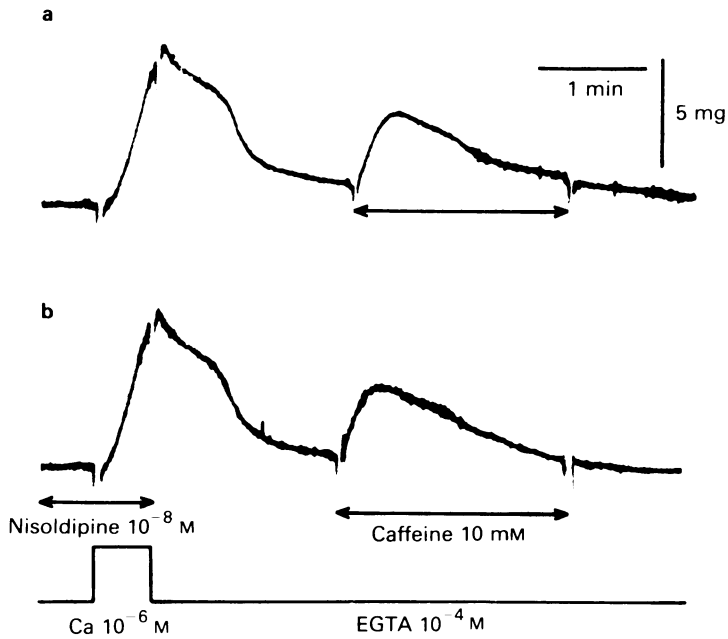

Figure 13 Effects of nisoldipine on the Ca accumulation and Ca release estimated from amplitude of the caffeine-induced contraction in skinned muscles. After application of Ca 10^{-6}M for 30 s, the tissue was rinsed with Ca-free relaxing solution (Methods) and caffeine 10 mM was applied. To observe the effects of nisoldipine, Ca 10^{-6} M and nisoldipine 10^{-8}M were applied simultaneously.

Figure 11 shows the effects of nisoldipine on the membrane potential in Na-free or Na- and Ca-free solution. (a and b) Were recorded from two different tissues. When Na was completely removed, the membrane was depolarized (up to 130 min in a and to 120 min in b), and successively applied Na- and Ca-free solution further depolarized the membrane in the presence (b) or absence (a) of 10^{-7} M nisoldipine. When the tissues were again superfused with Krebs solution, the membrane was transiently hyperpolarized within 10 min and restored to the control levels.

Effects of nisoldipine on the contractile protein and Ca storage sites

The effects of nisoldipine on the contractile protein and Ca storage site were investigated in saponin-treated skinned muscle tissues (Figure 12). Before application of saponin (25 μg ml^{-1}), 128 mM K was applied to evoke the contraction, after which the solution was replaced with the relaxing solution containing 25 μg ml^{-1} saponin for 20 min. When 10^{-6} M Ca was applied after the saponin containing solution had been replaced with the relaxing solution, the 10^{-6} M Ca-induced contraction reached the maximum value within 5 min (at 3 min, the amplitude was about 80% of the peak). When 10^{-8} M nisoldipine was applied with 10^{-6} M Ca after the contraction reached a steady level, the amplitude of the Ca-induced contraction was not affected. The effects of nisoldipine (10^{-8} M) on the pCa-tension relationship were also observed (Figure 12b). The Ca-induced contraction appeared with application of 10^{-7} M Ca (0.14 ± 0.05 times the maximum amplitude of contraction evoked by application of 4×10^{-6} M Ca, $n = 5$), and 3×10^{-7} M Ca produced 0.71 ± 0.04 times the maximum amplitude ($n = 5$). These Ca sensitivities of the skinned muscle tissue from the rabbit coronary artery were higher than those observed in the rabbit and guinea-pig mesenteric arteries (Itoh *et al.*, 1981; 1983a). Nisoldipine did not inhibit the pCa-tension relationship.

To observe the effects of nisoldipine on Ca accumulation into the storage site, the amount of stored Ca was estimated from the amplitude of the caffeine-induced contraction evoked after treatment with 10^{-6} M Ca in skinned muscle tissues (Figure 13). After skinning the tissue, Ca 10^{-6} M was applied for 30 s, then the tissue was rinsed with Ca-free (0.1 mM EGTA containing solution) for 2 min. When 10 mM caffeine was applied after the tissue had relaxed completely, contraction occurred (a). With application of nisoldipine (10^{-8} M) during treatment with

10^{-6}M Ca, the resulting caffeine-induced contraction was not affected (b). Nisoldipine, therefore, did not modify the Ca accumulation into the storage site as estimated from the amplitude of the caffeine-induced contraction.

Discussion

The K-induced contraction was generated mainly by activation of the voltage-dependent influx of Ca, because the K-induced contraction ceased promptly in Ca-free 2 mM EGTA-containing solution. However, the ACh-induced contraction was due to Ca-influx and release of Ca from the storage site. In Ca-free EGTA containing solution, ACh still produced a contraction with a slight reduction in the amplitude. Nisoldipine did not modify the contraction evoked by ACh or caffeine in Ca-free solution in the intact muscle, or Ca accumulation into the storage site, as estimated from caffeine-induced contraction in skinned muscles.

We postulated tentatively that the phasic response of the K-induced contraction is evoked by release of Ca from the storage site following activation of the influx of Ca (the Ca-induced Ca-release mechanism), and that the tonic response is generated by the amount of free Ca, balanced by the Ca influx, release and uptake at the storage site and also the Ca efflux. The influx of Ca, activated by the depolarization of the membrane, may not be instantaneously inhibited by nisoldipine but this agent may inhibit the voltage-dependent Ca channel after activation of the channel by depolarization. Therefore, when nisoldipine was applied simultaneously with 2.6 mM Ca and 128 mM K, the phasic contraction was evoked but when nisoldipine was applied with 2.6 mM Ca, following equilibration with 128 mM K, the phasic response was not evoked. Furthermore, accumulation of Ca into the storage site (estimated from the caffeine-induced contraction in Ca-free solution) preceded generation of the Ca-induced contraction.

The phasic response of the ACh-induced contraction may also be evoked by influx of Ca and release of Ca from the store site. Nisoldipine (5×10^{-8}M or 10^{-7}M) inhibited the oscillatory potential changes superimposed on the slow depolarization evoked by ACh, but this agent (below 10^{-7}M) did not inhibit the slow depolarization. If the phasic response of the ACh-induced contraction was evoked solely by membrane activity, this response may be inhibited by nisoldipine, but such was not the case. Therefore, the phasic response of the ACh-induced contraction is evoked by nisoldipine-resistant Ca influx and Ca release. In addition, the Ca-induced Ca release mechanism may occur under conditions related to the amount of Ca storage. In the pig and rabbit coronary arteries, ACh contracted intact muscle tissue in Ca-free solution, while ACh itself did not act on the contractile proteins and Ca storage sites in the skinned muscles (Itoh *et al.*, 1982; Kanmura *et al.*, 1983b). Presumably, ACh produces a Ca releasing substance (e.g. phosphatidyl inositol break down) by activation of the muscarinic ACh-receptor (Michell, 1975; Michell *et al.*, 1976). Therefore, if the amount of stored Ca is adequate, nisoldipine does not inhibit the phasic response of the ACh-induced contraction and the inhibition of the tonic response is smaller than that observed in the K-induced contraction. Since the voltage-dependent Ca influx is only a part of the Ca influx activated by ACh, the concentration of free Ca resulting from Ca influx, Ca release, Ca re-uptake and Ca extrusion in the presence of nisoldipine may be higher than that in the case of the K-induced contraction.

In various visceral and vascular smooth muscles, the intracellular Na concentration becomes almost nil in Na-free solution after 30 min superfusion (Brading, 1981; Casteels, 1981; Jones, 1980; 1981). Reduction of Ca extrusion or increase in the Ca influx caused by Na-free solution may contribute to generation of the contraction (Blaustein, 1977; Brading, 1979; 1981; Casteels, 1981; Itoh *et al.*, 1983a,b); however, the Na-free induced contraction was not observed in the porcine coronary artery (Hirata *et al.*, 1981). In Na-free solution, smooth muscles of the rabbit coronary artery produce a contraction, and reversion to the control level occurred within 60 min. Following superfusion with Na- and Ca-free solution, application of Ca (2.6 mM) produced contraction which was blocked by treatment with nisoldipine. In Na-free solution, the membrane was depolarized, thereby contributing to the contraction. The contraction evoked in Na-free solution under the present experiments may not be due to the Na (efflux)-Ca (influx) exchange diffusion, but rather to the voltage-dependent Ca influx. However, contributions of the Na-Ca exchange diffusion may not be ruled out completely for the contraction was evoked just after application of Na-free solution.

It has also been reported that prolonged superfusion with Na-free solution inhibits Ca extrusion from cells, and as a consequence, the accumulation of Ca in cells is increased (Itoh *et al.*, 1983a). The caffeine-induced contraction in Na- and Ca-free solution, therefore, showed a larger amplitude and repetitive application of caffeine produced successively generated contractions, as compared to the findings in Ca-free Na containing solution. Application of nisoldipine inhibited the Ca-induced contraction but did not inhibit the caffeine-induced contraction in Ca- and Na-free medium, because nisoldipine did not inhibit the release of Ca stored in cells by the action of caffeine.

The actions of nisoldipine on the rabbit coronary artery can be summarized as follows; the phasic response of the K-induced contraction requires increase in the influx of Ca and activation of the Ca-induced Ca-release mechanism, thus rapidly increasing the free Ca in the myoplasm, with the condition that a certain amount of Ca has already been stored. The tonic response of the K-induced contraction depends on the amount of Ca influx (including the Ca release from the storage site), Ca re-uptake into the storage site and Ca extrusion. Therefore, inhibition of the Ca influx by nisoldipine may predominantly inhibit the tonic rather than the phasic response. This agent does not inhibit the phasic response of the ACh receptor activated contraction because, nisoldipine inhibits the active response of the membrane but not all the Ca influx activated by ACh. Therefore, the tonic response is preserved to a greater extent than that evoked by 128 mM K, under the conditions of Ca storage. In the polarized muscles, nisoldipine does not inhibit the increase in the Ca influx during application of Ca in Ca-free solution. This agent has no effect on contractile proteins including calmodulin, or the Ca accumulation into and Ca release from the storage site. Nisoldipine, therefore, selectively inhibits the voltage-dependent Ca influx only when the Ca channel is activated by depolarization.

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References

ABE, Y. & TOMITA, T. (1968). Cable properties of smooth muscles. *J. Physiol.*, **196,** 87–100.

BLAUSTEIN, M.P. (1977). Sodium ions, calcium ions, blood pressure regulation and hypertension: a reassessment and a hypothesis. *Am. J. Physiol.*, **232,** c165–c173.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59,** 606–718.

BRADING A.F. (1979). Maintenance of ionic composition. *Br. med. Bull.*, **35,** 227–234.

BRADING A.F. (1981). Ionic distribution and mechanism of transmembrane ion movements in smooth muscle. In *Smooth Muscle.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

CASTEELS, R. (1981). Membrane potential in smooth muscle. In *Smooth Muscle.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita. T. pp. 105–126. London: Edward Arnold.

CAUVIN, C., LOUTZENHISER, R. & VANBREEMEN, C. (1983). Mechanisms of calcium vasodilation. *A. Rev. Pharmac. Tox.*, **23,** 373–396.

FLECKENSTEIN, A. (1977). Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. *A. Rev. Pharmac. Tox.*, **17,** 149–166.

FLECKENSTEIN, A. (1983). *Calcium Antagonism in Heart and Smooth Muscle.* New York: Wiley-Interscience.

GOLENHOFEN, K. (1981). Differentation of calcium activation processes in smooth muscle using selective antagonists. In *Smooth Muscle.* ed, Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 157–197. London: Edward Arnold.

GRUN, G. & FLECKENSTEIN, A. (1972). Die elektromechaniscke Entkoppelung der glatten Gefa muskulatue als Grundprinzip der koronardilatation durch 4-(2'-Nitrophenyl)-2,6-dimetyl-1, 4-dihydropyridin-3, 5-dicarbonsanredimethylester (BAY a 1040, nifedipine). *Arzn. Forsch.* (Drug Res.), **22,** 334–344.

HIRATA, M., ITOH, T. & KURIYAMA, H. (1981). Effects of external cations on calcium efflux from single cells of the guinea-pig taenia coli and porcine coronary artery. *J. Physiol.*, **310,** 321–336.

ITOH, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1982). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *J. Physiol.*, **322,** 107–125.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **321,** 513–535.

ITOH, T., KURIYAMA, H. & SUZUKI, M. (1983a). Differences and similarities in the noradrenaline-and caffeine-induced mechanical responses in the rabbit mesenteric artery. *J. Physiol.*, **337,** 609 629.

ITOH, T., KURIYAMA, H. & UENO, H. (1983b). Mechanisms of the nitroglycerine-induced vasodilation in vascular smooth muscle of the rabbit and pig. *J. Physiol.*, **343,** 233–252.

JATENE, A.D. & LICHTLEN, P.R. (1976). Third International Adalat Symposium: *New Therapy of Ischemic Heart Disease.* Amsterdam–Oxford: Excerpta Medica.

JOHNSSON, B. & SOMLYO A.P. (1980). Electrophysiology and excitation contraction coupling. In *Handbook of Physiology: The Cardiovascular System,* sect. 2, vol. II. ed. Bohr, D.F., Somlyo, A.P. & Sparks, H.D. Jr. pp. 301–323. Bethesda, MD: American Physiological Society.

JONES, A.W. (1980). Content and fluxes of electrolytes. In *Handbook of Physiology,* sect. 2, vol. II, *The Cardiovascular System.* ed. Bohr, D.F., Somlyo, A.P. & Sparks, H.D. Jr. pp. 253–299. Bethesda, MD: American Physiological Society.

JONES A.W. (1981). Vascular smooth muscle and alterations during hypertension. In *Smooth Muscle.* ed. Bülbring, E., Brading A.F., Jones, A.W. & Tomita, T. pp. 397–430. London: Edward Arnold.

KANMURA, Y., ITOH, T., SUZUKI, H., ITO, Y. & KURIYAMA, H. (1983a). Effects of nifedipine on smooth muscle cells of the rabbit mesenteric artery. *J. Pharmac. exp. Ther.*, **226,** 238–248.

KANMURA, Y., ITOH, T., SUZUKI, H., ITO, Y. & KURIYA-

MA, H. (1983b). Nifedipine actions on smooth muscle cells of pig and rabbit skinned and intact coronary arteries. In *Asian-Pacific Adalat Symposium* ed. Hashimoto, K. & Kawai, C. pp. 3–30. Tokyo: Medical Tribune Inc.

KURIYAMA, H., ITO, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying the contraction-relaxation cycle in vascular smooth muscles. *Am. J. Physiol.*, **243,** H641–H662.

MAKITA, Y., KANMURA, Y., ITOH, T., SUZUKI, H. & KURIYAMA, H. (1983). Effects of nifedipine derivatives on smooth muscle cells and neuromuscular transmission in the rabbit mesenteric artery. *Naunyn-Schmiedebergs Arch. Pharmac.*, **324,** 302–312.

MICHELL, R.H. (1975). Inositol phospholipids and cell surface receptor function. *Biochem. biophys. Acta*, **415,** 81–147.

MICHELL, R.H., JAFFERJI, S.S. & JONES, L.M. (1976). Receptor occupancy dose-response curve suggests that phosphatidyl inositol breakdown may be intrinsic to the mechanism of the mesenteric cholinergic receptor. *FEBS Letters*, **69,** 1–5.

PUECH, P. & KREBS, R. (1980). *Fourth International Adalat Symposium: New Therapy of Ischemic Heart Disease.* Amsterdam–Oxford–Princeton: Excerpta Medica.

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