Blockade by calmodulin inhibitors of Ca^{2+} channels in smooth muscle from rat vas deferens

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1 Effects of three compounds which are used as calmodulin inhibitors (trifluoperazine, W-7 and calmidazolium) on Ca^{2+} channels were investigated in smooth muscle from rat vas deferens.

2 All three calmodulin inhibitors relaxed the smooth muscle precontracted by a high concentration of KCl (63.7 mM). The order of potency for the relaxation was trifluoperazine > W-7 > calmidazolium.

3 In binding studies using a microsomal fraction of vas deferens, all these calmodulin inhibitors displaced specific [^3H]-nimodipine binding. Trifluoperazine and W-7 inhibited the binding at concentrations that relaxed the smooth muscle whereas calmidazolium inhibited at concentrations much lower than those necessary for muscle relaxation.

4 Ba^{2+} current flowing through voltage-gated Ca^{2+} channels was measured under whole-cell voltage-clamp conditions in isolated smooth muscle cells. The Ba^{2+} current was suppressed by the three calmodulin inhibitors in the concentration-range where inhibition of [^3H]-nimodipine binding was observed. Neither voltage-dependence nor the inactivation time course of Ba^{2+} current were affected by these compounds.

5 The results suggest that the calmodulin inhibitors directly block Ca^{2+} channels in the smooth muscle cells. The channel inhibition by trifluoperazine and W-7, but perhaps not that by calmidazolium, may be responsible for the muscle relaxation observed with these compounds.

Keywords: Vas deferens; smooth muscle; calmodulin inhibitors; calcium channel; dihydropyridine binding; voltage-clamp

Introduction

Calmodulin is an intracellular Ca^{2+} -receptive protein that exhibits various functions (Cheung, 1980; Tanaka, 1988). Calmodulin also plays a role in smooth muscle contraction (Kreye *et al.*, 1983; Hidaka *et al.*, 1979; 1980; Tanaka, 1988). Agents that bind to calmodulin and block its functions have been developed (e.g., Gietzen *et al.*, 1981; Hidaka *et al.*, 1980; 1981; Tanaka, 1988). These calmodulin inhibitors have been widely used as pharmacological tools to test whether biological responses involve calmodulin-dependent mechanisms (e.g. Bkaily *et al.*, 1984; Campbell *et al.*, 1986; Koyama & Himori, 1988). However, it has been suggested that these compounds exert effects that are not related to calmodulin inhibition when applied extracellularly (e.g., Klöckner & Isenberg, 1987; Hori *et al.*, 1989). Inhibition of Ca^{2+} channels by these compounds was also investigated by electrophysiological techniques in cardiac cells (Klöckner & Isenberg, 1987) and in *Paramecium* (Ehrlich *et al.*, 1988). Klöckner & Isenberg (1987) reported that several calmodulin inhibitors reduced Ca^{2+} currents in smooth muscle cells from bovine portal vein. Effects of calmodulin inhibitors on Ca^{2+} channels in non-vascular smooth muscles were not reported. In addition, it is not known whether the channel inhibition leads to inhibition of smooth muscle contraction.

The present study was aimed to determine effects of calmodulin inhibitors on Ca^{2+} channels in smooth muscles from rat vas deferens. We found that three calmodulin inhibitors, trifluoperazine, W-7 and calmidazolium, relaxed the smooth muscle contracted by a high concentration of KCl, inhibited specific [^3H]-nimodipine binding, and Ba^{2+} current permeating through Ca^{2+} channels.

Methods

Measurement of muscle tension

Male Wistar strain rats (250 to 350 g) were killed by cervical dislocation and exsanguination. The vas deferens was dissected and surrounding blood vessels and adipose tissue were removed. Isometric tension of the longitudinal muscles was recorded under the conditions described previously (Higo *et al.*, 1988; Abe *et al.*, 1989). Briefly, the vas deferens was mounted in a 10 ml organ bath chamber filled with a solution of the following composition (in mM): NaCl 118, KCl 4.7, CaCl_2 1.8, MgCl_2 1.2, NaH_2PO_4 1.2, NaHCO_3 25.0, glucose 11.1 (oxygenated with 95% O_2 :5% CO_2 ; pH 7.4 at 37°C). The tissue was allowed to equilibrate for 60 min. After the equilibrium period, tonic contraction was produced by a solution containing 63.7 mM KCl (NaCl in the standard solution was replaced with equimolar KCl). Prazosin (10^{-6} M) was included in this solution to prevent any contribution of endogenous noradrenaline. Concentration-response curves for muscle relaxation were obtained by cumulative addition of calmodulin inhibitors. The tension was measured with a force-displacement transducer (Nihon Kohden TB-612T) and recorded on an ink-writing oscillograph (Nihon Kohden WI621-G).

Radioligand binding assay

Interaction of calmodulin inhibitors with dihydropyridine binding was determined according to Higo *et al.* (1989). Briefly, a microsomal fraction was prepared from rat vas deferens, and suspended in 0.2 mM of Tris-HCl buffer (pH 7.4). The protein content in the suspension was adjusted to 0.5–1.0 mg ml $^{-1}$. The suspension was incubated with 0.3 nM [^3H]-nimodipine in the presence of various concentrations of calmodulin inhibitors at 25°C for 20 min in a duplicate set of tubes. The incubation was terminated by filtration through Whatman GF/F filters. The radioactivity

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remaining on the filters was counted with a liquid scintillation spectrometer (Aloka LSC-700). Specific binding of [3 H]-nimodipine was defined as the binding displaceable by 10 μ M of unlabelled nimodipine. Data were fitted by curves according to a conventional drug-receptor theory (Tallarida & Jacob, 1979). The best fit was determined with a least squares method from the formula: $\ln[B/(B_{\max}-B)] = -n_H \times [\ln A - \ln(IC_{50})]$, where A is a concentration of drugs, B is specific binding of [3 H]-nimodipine, and n_H is the Hill coefficient.

Membrane current recordings

Membrane currents were recorded from enzymatically dispersed single smooth muscle cells from rat vas deferens (Nakazawa *et al.*, 1987). Electrical recordings were made by the whole-cell voltage-clamp technique (Hamil *et al.*, 1981; Marty & Neher, 1983) under the conditions described elsewhere (Nakazawa *et al.*, 1987; 1988). The isolated cells were continuously superfused with an extracellular solution containing (in mM): NaCl 140, BaCl₂ 10.8, MgCl₂ 1.2, HEPES 10, glucose 11.1 (pH 7.4 with NaOH). We used 10.8 mM Ba²⁺ as a divalent charge carrier to obtain a large inward current through Ca²⁺ channels (Nakazawa *et al.*, 1988). Heat-polished patch pipettes had tip resistance of 2 to 4 Mohm when filled with an intracellular solution containing (in mM): CsCl 130, succinic acid 5, oxalacetic acid 5, pyruvic acid 5, HEPES 10, EGTA 2, MgCl₂ 1, CaCl₂ 0.18 (pH 7.2 with CsOH). The electrical signals were recorded with a patch-clamp amplifier (Nihon Kohden S-3666) and stored on magnetic tape for later analysis. The current traces were digitized and analysed on a personal computer (NEC PC9801). A linear component of leak current was estimated from a current response to a hyperpolarizing step from -60 mV to -100 mV and this component was subtracted from current traces obtained with depolarizing steps. Current measurements were carried out at room temperature (about 25°C).

Chemicals

Trifluoperazine dihydrochloride, W-7 (N-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide) hydrochloride, calmidazolium (compound R24571) and nicardipine hydrochloride were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Agents used in the binding assays were [3 H]-nimodipine (specific activity: 138.5 Ci mmol⁻¹, Dupon-New England Nuclear, Boston, MA, U.S.A.) and nimodipine (Bayer, Lerverkusen, Germany). Trifluoperazine and W-7 were first dissolved in dimethyl sulphoxide (DMSO) and added to experimental solutions. Final concentrations of DMSO were less than 1%, which did not affect the muscle tension, [3 H]-nimodipine binding or Ca²⁺ current described here.

Results

Muscle relaxation induced by calmodulin inhibitors

Figure 1 shows effects of calmodulin inhibitors on smooth muscles contracted by 63.7 mM KCl. The tonic contraction was relaxed by cumulative application of the calmodulin inhibitors in a concentration-dependent manner. Trifluoperazine and W-7 completely relaxed the smooth muscle at the highest concentrations tested (Figure 1). The concentration-response curves suggests the order of potency was trifluoperazine > W-7. Calmidazolium also relaxed the muscles. The potency of calmidazolium was weaker than that of W-7; higher concentrations could not be tested.

Inhibition of [3 H]-nimodipine binding

We previously showed that [3 H]-nimodipine bound to a microsomal fraction prepared from smooth muscle of rat vas

deferens in a saturable and reversible manner, and that other dihydropyridine Ca²⁺ channel antagonists inhibited the [3 H]-nimodipine binding (Higo *et al.*, 1988). A test was made to determine whether the calmodulin inhibitors affect [3 H]-nimodipine binding. As shown in Figure 2, all the calmodulin inhibitors inhibited [3 H]-nimodipine binding, trifluoperazine and W-7 inhibiting it at concentrations that relaxed the smooth muscles (Figure 1). IC₅₀ values calculated were 0.40 μ M for trifluoperazine and 12.6 μ M for W-7. Low concentrations (10⁻⁷ to 10⁻⁵ M) of calmidazolium, which scarcely affected the smooth muscle tension (Figure 1), inhibited [3 H]-nimodipine binding (IC₅₀ was 0.25 μ M).

Figure 1 Relaxation by trifluoperazine, W-7 and calmidazolium of smooth muscles from rat vas deferens. The smooth muscles were contracted by a solution containing 63.7 mM KCl. W-7 (●), trifluoperazine (○) and calmidazolium (▲) were added during the tonic phase of contraction in a cumulative manner. Each point and vertical bar represent the mean and s.e.mean from five experiments.

Figure 2 Inhibition of [3 H]-nimodipine binding by W-7 (●), trifluoperazine (○) and calmidazolium (▲). The drugs were incubated with the microsomal membranes of rat vas deferens in the presence of 0.3 nM [3 H]-nimodipine for 20 min at 25°C. Specific binding was determined as the fraction that was displaced by 10⁻⁵ M of unlabelled nimodipine. Each symbol represents the mean $\pm$ s.e.mean from 3–8 preparations. Smooth curves were best-fitted to the data as described in Methods using following values: n_H = 0.78 (W-7), 0.77 (trifluoperazine) or 0.78 (calmidazolium); IC₅₀ = 12.6 (W-7), 0.4 (trifluoperazine) or 0.25 μ M (calmidazolium).

Blockade of Ba²⁺ current flowing through Ca²⁺ channels

Ba²⁺ current flowing through voltage-gated Ca²⁺ channels was measured to determine whether the calmodulin inhibitors affect functional channels. Trifluoperazine and W-7 inhibited the Ba²⁺ current (Figures 3, 4 and 5) at concentrations that relaxed the smooth muscles (Figure 1) and inhibited the [³H]-nimodipine binding (Figure 2). Calmidazolium suppressed the Ba²⁺ current (Figures 3 and 5) at concentrations (10⁻⁷ to 10⁻⁶ M) that scarcely affected the smooth muscle tension (Figure 1) and markedly inhibited [³H]-nimodipine binding (Figure 2). The recovery from the inhibition was different among the calmodulin inhibitors (Figure 3). The Ba²⁺ current suppressed by trifluoperazine (10⁻⁶ M) was almost fully recovered after a 5 min rinse with a drug-free solution. The current inhibited by W-7 (10⁻⁴ M) or calmidazolium (10⁻⁷ M) was partially recovered after a 5 min rinse: the peak Ba²⁺ current recovered was about 70% (W-7) or 40% (calmidazolium) of the current before administration. Figure 4 shows the current-voltage relationship for the Ba²⁺ current. Trifluoperazine (10⁻⁷ M, Figure 4a), W-7 (3 × 10⁻⁵ M, Figure 4b) or calmidazolium (10⁻⁷ M, not shown) did not affect the voltage-dependence of the Ba²⁺ current.

Dihydropyridine Ca²⁺ channel antagonists are known to accelerate inactivation of L-type Ca²⁺ channels (Lee & Tsien, 1983). We previously reported that such acceleration was also observed with nicardipine in smooth muscle cells from rat vas deferens (Nakazawa *et al.*, 1988). To determine the effects of the calmodulin inhibitors on the inactivation kinetics of the Ba²⁺ current, we compared the amplitude of peak Ba²⁺ current and that of the current remaining after 400 ms (Figure 5). In this comparison, the Ba²⁺ current remaining after 400 ms was suppressed by nicardipine (10⁻⁷ M) to a significantly larger extent than the peak Ba²⁺ current (Figure 5). The Ba²⁺ current remaining after 400 ms was also somewhat more sensitive to any of three calmodulin inhibitors than the peak Ba²⁺ current, but the difference was not statistically significant (Student's *t* test, *P* > 0.05). The results suggest that the calmodulin inhibitors do not affect the inactivation kinetics as distinctly as dihydropyridine Ca²⁺ antagonists do.

Effects of preincubated calmidazolium on Ca²⁺-induced contraction

As described above, [³H]-nimodipine binding (Figure 2) and Ba²⁺ current permeating through Ca²⁺ channels (Figures 3 and 5) were markedly inhibited by calmidazolium at 10⁻⁷–10⁻⁵ M whereas smooth muscle precontracted with 63.7 mM KCl was scarcely relaxed in this concentration-range (Figure 1). Spedding (1982) assessed effects of compounds

that exert Ca²⁺ antagonistic activity in guinea-pig taenia caecum, and found that a subclass of such compounds fail to relax precontracted smooth muscle though they potentially inhibit Ca²⁺-induced contraction when applied initially in Ca²⁺-free media. We tested whether preincubation in Ca²⁺-free media potentiates the inhibition by calmidazolium. In this experiment, smooth muscle was bathed in a Ca²⁺-free solution containing 63.7 mM KCl, and contraction was achieved by addition of 1.8 mM Ca²⁺. However, preincuba-

Figure 4 Current-voltage relationship for Ba²⁺ current flow through Ca²⁺ channels in the absence and presence of trifluoperazine (10⁻⁷ M, a) or W-7 (3 × 10⁻⁵ M, b). The cells were held at -60 mV and 400 ms steps to various test potentials were applied every 10 s. Each symbol represents peak Ba²⁺ current just before (●) and 2 min after (○) application of drugs. (a) and (b) Were obtained from different cells.

Figure 3 Blockade by trifluoperazine (10⁻⁶ M, a) or calmidazolium (10⁻⁷ M, b) of Ba²⁺ current flowing through voltage-gated Ca²⁺ channels in smooth muscle cells isolated from rat vas deferens. The cells were bathed in an extracellular solution containing 10.8 mM Ba²⁺ and held at -60 mV. The Ba²⁺ current was activated by applying a voltage step to +10 mV. Traces are the Ba²⁺ current just before (left) and 2 min after drug-application (middle), and the current after a 5 min rinse with a drug-free solution (right). (a) and (b) Were obtained from different cells.

Figure 5 Blockade by trifluoperazine, W-7 and calmidazolium of peak Ba^{2+} current (open columns) and the Ba^{2+} current measured after 400 ms (hatched columns). The effects of nicardipine are also illustrated for comparison. The Ba^{2+} current was activated by a depolarizing step to +10 mV from a holding potential of -60 mV. The current amplitude 2 min after administration of drugs was normalized to the amplitude just before the administration. Each column represents the mean $\pm$ s.e. mean. The number of cells tested is indicated in parentheses. *Statistically significant difference from inhibition of the peak Ba^{2+} current (Student's *t* test, $P < 0.05$).

tion with calmidazolium (10^{-6} M) did not significantly inhibit smooth muscle contraction, the difference between the contraction in control condition and that with calmidazolium (10^{-6} M) being less than 10% ($n = 2$).

Discussion

We have investigated the effects of three compounds which have been used as calmodulin inhibitors on Ca^{2+} channels in smooth muscles from rat vas deferens. These compounds blocked the Ba^{2+} current flowing through Ca^{2+} channels (Figures 3, 4 and 5) at concentrations similar to those required for the inhibition of ionic currents through Ca^{2+} channels in other cells (Klößner & Isenberg, 1987; Ehrlich *et al.*, 1988). Moreover, the calmodulin inhibitors inhibited [^3H]-nimodipine binding (Figure 2). Recent investigations have clarified that dihydropyridine binding sites exist on the pore-forming subunit of L-type Ca^{2+} channels (α_1 -subunit; Miller, 1992). Thus, the inhibition of [^3H]-nimodipine binding suggests that the calmodulin inhibitors affect the Ca^{2+} channels themselves or compartments closely coupled with the channels, and may exclude the possibility that some second messenger such as calmodulin mediates the channel inhibition.

Trifluoperazine and W-7 relaxed the smooth muscle contracted by a high concentration of KCl (Figure 1). The contraction may be due to Ca^{2+} -influx through L-type Ca^{2+}

channels because both the contraction (Higo *et al.*, 1988) and the current flowing through Ca^{2+} channels (Nakazawa *et al.*, 1987; 1988) are completely blocked by dihydropyridine Ca^{2+} channel antagonists. Thus, the relaxation by trifluoperazine or W-7 may also result from their inhibitory effect on the Ca^{2+} channels. Similarity between magnitude of the relaxation (Figure 1) and that of the current inhibition (Figure 5) may support this idea. In contrast, calmidazolium markedly inhibited the Ba^{2+} current (Figures 3, 4 and 5) without affecting the muscle tension (Figure 1) at concentrations lower than 10^{-7} M. This finding is rather paradoxical. Perhaps, the muscle relaxation which is expected from the blockade of the Ca^{2+} channels may be hampered by some unknown mechanisms. Protective effects of Ca^{2+} against Ca^{2+} antagonistic effects which have been reported for compounds such as flunarizine or cinnarizine (Spedding, 1982) may not contribute to the lack of significant blockade by calmidazolium because application of this compound to Ca^{2+} -free media did not markedly affect the contraction, either.

Thayer & Fairhurst (1983) reported that nitrendipine bound to calmodulin in rat brain. It may be argued that [^3H]-nimodipine binding in the present study comprises such binding to calmodulin. However, this may not be the case because the [^3H]-nitrendipine binding to calmodulin in rat brain was of low affinity and unsaturable (Thayer & Fairhurst, 1983) whereas the [^3H]-nimodipine binding in rat vas deferens was of a high affinity ($K_d = 0.31$ nM) and saturable (Higo *et al.*, 1988). The [^3H]-nimodipine binding sites in this paper may be best interpreted as L-type Ca^{2+} channels (Reynolds & Snyder, 1988).

We cannot conclude at present whether the blockade by the calmodulin inhibitors of [^3H]-nimodipine binding is due to displacement at the same binding site or some allosteric effect. Ehrlich *et al.* (1988) reported that W-7 inhibited 'T-type' Ca^{2+} channels isolated from *Paramecium*. Klößner & Isenberg (1987) reported that trifluoperazine and calmidazolium inhibited Na^+ and K^+ channels in cardiac or vascular smooth muscle cells at concentrations about 10 fold higher than those necessary for Ca^{2+} channel inhibition. The blockade of these 'dihydropyridine-insensitive' channels may support the idea of an allosteric effect. One possibility is that the binding of the calmodulin antagonists induces a conformational change in the Ca^{2+} channels so that dihydropyridine binding as well as channel opening is inhibited.

Levin & Weiss (1979) and Itoh *et al.* (1986) studied the binding of calmodulin inhibitors to calmodulin. When K_i values were calculated from their data using the equation of Chang & Prusoff (1973), K_i values were $0.62 \mu\text{M}$ for trifluoperazine (Levin & Weiss, 1979) and $26 \mu\text{M}$ for W-7 (Itoh *et al.*, 1986). These values are comparable to IC_{50} values obtained from the blockade of [^3H]-nimodipine binding ($0.40 \mu\text{M}$ for trifluoperazine and $12.6 \mu\text{M}$ for W-7; Figure 2) and concentrations necessary for the muscle relaxation (Figure 1) or the inhibition of the Ba^{2+} current (Figures 3, 4 and 5). As for calmidazolium, the IC_{50} for inhibition of calmodulin-dependent ATPase was $0.35 \mu\text{M}$ (Gietzen *et al.*, 1981). This value is also comparable to the concentrations necessary for the blockade of [^3H]-nimodipine binding (Figure 2) or the Ba^{2+} current (Figures 3 and 5). Therefore, these compounds cannot inhibit calmodulin-mediated responses without affecting the Ca^{2+} channels when applied extracellularly.

In conclusion, we have demonstrated that three calmodulin inhibitors act as antagonists to L-type Ca^{2+} channels in smooth muscle cells from rat vas deferens, and that the channel inhibition by trifluoperazine and W-7, but not that by calmidazolium, can account for the relaxations of smooth muscle contracted with a high concentration of KCl. These compounds cannot be used as pure calmodulin inhibitors because the drugs directly inhibit Ca^{2+} channels.

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(Received September 10, 1992

Revised December 14, 1992

Accepted January 8, 1993)