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Ca^{2+}-entry blockade by CAF603, a carotane sesquiterpene isolated from *Trichoderma virens*

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Abstract

Isometric tension recordings and patch-clamp methods were combined to explore the functional effects and mechanisms of action of 8-daucene-3,4-diol (CAF603), a carotane sesquiterpene isolated from the fungus *Trichoderma virens*. CAF603 (1–100 μM) inhibited the spontaneous motility of guinea-pig portal vein, duodenum and ileum, and the Ca^{2+}-induced tension of depolarized ileum strips. These effects were not antagonized by either iberiotoxin or glyburide. CAF603 increased the spontaneous motility of guinea-pig detrusor muscle, but inhibited the contraction induced by high-KCl, depolarizing salines. CAF603 blocked L-type Ca^{2+} channel currents of rabbit cardiac myocytes. It is proposed that Ca^{2+}-entry blockade accounts for the inhibitory effects of CAF603 on smooth muscle contractility, whereas the stimulation of spontaneous motility of detrusor muscle is ascribed to blockade of Ca^{2+}-activated K^+ (BK_{Ca}) channel currents. The latter interpretation is consistent with the allosteric modulation of charybdotoxin binding to BK_{Ca} in smooth muscle membranes [Lee et al., 1995. J. Nat. Prod. 58, 1822–1828]. © 1997 Elsevier Science B.V.

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1. Introduction

Pharmacological modulation of ionic channels has been the focus of a great deal of attention during recent years, in attempts to discover potential targets for drug development. Large scale, high-capacity screening assays of natural products have been successfully used to identify such targets. This is well illustrated for the case of high-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, modulators of which have been discovered by means of a [^{125}I]charybdotoxin binding screen (Vazquez et al., 1989). Thus, from extracts of *Desmodium adscendens*, a medicinal herb used in Ghana, McManus et al. (1993) purified three glycotriterpenes (soyasaponins), which are potent BK_{Ca} channel agonists. Other modulators of this channel revealed by the [^{125}I]charybdotoxin binding assay include the tremorgenic indole alkaloids, paxilline and paspalitrem-A and C, which act as channel antagonists (Knaus et al., 1994; DeFarias et al., 1996), the agonist maxikdiol, extracted from an unidentified coelomycete (Singh et al., 1994) and the sesquiterpene CAF603 (8-daucene-3,4-diol; Fig. 1), isolated from strains of *Trichoderma virens* (Ondeyka et al., 1995).

This latter compound, which is the object of the present study, despite its ability to block [^{125}I]charybdotoxin binding to sarcolemmal membranes, showed no clear effect on BK_{Ca} channel currents recorded with the patch-clamp technique (Lee et al., 1995). Preliminary functional studies, carried out in parallel with these electrophysiological experiments, revealed that CAF603 reduced the spontaneous motility of rat's portal vein, while increasing the contractility of urinary bladder detrusor muscle (Suarez-Kurtz, unpublished). In the present study we have con-

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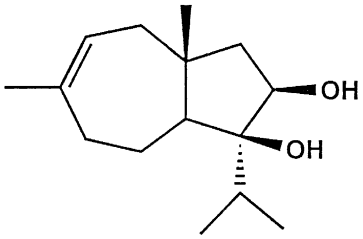

Fig. 1. Chemical structure of CAF603.

firmed these observations in another species, namely the guinea pig, and extended our investigation of the functional effects of CAF603. Data obtained from a variety of preparations, using isometric tension and patch-clamp recordings reveal that the predominant functional effect of CAF603 in both smooth and heart myocytes is L-type Ca^{2+} channel blockade. CAF603, a carotene sesquiterpene, is the first representative of this novel chemical class of Ca^{2+}-entry blockers, distinct from phenylalkylamines, benzothiazepines and dihydropyridines, which are of important therapeutic value.

2. Materials and methods

2.1. Tension recordings from smooth muscle preparations

Experiments were performed at 37°C on tissues obtained from adult guinea pigs after death by ether inhalation. Preparations of portal vein and strips of duodenum, ileum and detrusor muscle from the urinary bladder were mounted between two metal stirrups, of which the lower was fixed and the upper attached to a rigid wire connected to a force-displacement transducer (Grass FT-03; Grass Instruments, Quincy, MA, USA). The transducer signals were amplified and recorded on a Grass polygraph (Model 7). The amplified signals from detrusor muscle, duodenum, ileum and portal vein, preparations which exhibited spontaneous motility were fed into an integrator (Grass 7P10) for quantification of the myogenic activity (Suarez-Kurtz et al., 1991). A 1 g load was initially applied to all preparations.

The physiological saline solution, a modified Krebs–Henseleit–Bicarbonate (KHB) solution had the following composition (in mmol): NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.1, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11 and Hepes 10. The pH of this solution after equilibration with 95% O_2 and 5% CO_2 was 7.3 at 37°C. Depolarizing, high-KCl solutions were prepared by iso-osmotic replacement of NaCl with KCl. A nominally Ca^{2+}-free, depolarizing solution, containing 43 mM KCl was used in some experiments with ileum strips.

2.2. Patch-clamp recordings of rabbit ventricular myocytes

Myocytes were enzymatically dissociated from ventricles of adult rabbits. After death by cervical dislocation, the heart was removed and the aorta was quickly cannulated for retrograde perfusion by the Langerdorff method (cf., Perez et al., 1984), at a constant flow of 10 ml/min, with KHB solution containing 1 mM $CaCl_2$. After blood had been washed out, the perfusate was changed to a nominally Ca^{2+}-free KHB solution, until cardiac arrest. Collagenase (331 U/mg, type CLS 2, Worthington Biochemical, Freehold, NJ) was then added (0.1–0.2 mg/ml) to the Ca^{2+}-free KHB solution and perfusion was continued for 10 to 20 min, followed by a washing period of 5 min with the Ca^{2+}-free KHB solution. The ventricles were chopped with scissors and stored until use in a high-K^+, low-Cl^- solution containing (in mM): KCl 30, glutamic acid 70, KH_2PO_4 10, $MgCl_2$ 1, taurine 20, EGTA 0.3, glucose 10, HEPES 10, pH adjusted to 7.3 with KOH. Dispersed cells were obtained by mechanical agitation of small pieces of ventricles. An aliquot of the cell suspension was added to the recording chamber and allowed to settle, under continuous superfusion at the rate of 1 ml/min with a Tyrode solution containing (in mM): NaCl 150.8, KCl 2.7, $MgCl_2$ 0.5, $CaCl_2$ 2.7, glucose 6, HEPES 10, pH 7.4. Single cells used in experiments were quiescent, rod-shaped with regular cross-striations, and did not have cytoplasmatic granules or membrane blebs.

Voltage-clamp experiments were performed at room temperature (25–27°C), in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using an Axopatch-1D amplifier (Axon Instruments, Burlington, CA). Micropipettes, with resistances from 2 to 5 MΩ, pulled from 1.2 mm o.d. glass tubing, were filled with a solution designed to eliminate K^+ currents, containing (in mM): CsCl 120, tetraethylammonium chloride 20, $MgCl_2$ 1, EGTA 5, HEPES 10, titrated to pH 7.4 with CsOH. In order to minimize rundown of the Ca^{2+} current, Na_2ATP (5 mM) was added to this solution.

Current signals were filtered with a 8-pole Bessel filter with a cutoff frequency of 2 kHz and digitized on-line at a sampling frequency of 5 kHz using a 12-bit 330 kHz A/D converter (Digidata 1200, Axon Instruments). Voltage clamp pulses, data acquisition, and data analysis were accomplished with a software package (pCLAMP 6, Axon Instruments) running on a 486 PC-compatible computer. Cells were clamped at a holding potential of −40 mV to inactivate both the fast Na^+ current and the low-threshold (T-type) Ca^{2+} current. The standard protocol consisted of applying step pulses of 300 ms duration, from −50 to +60 mV, with increments of 10 mV at 0.1 Hz. The L-type Ca^{2+} current was measured as the difference between the peak inward current and the current recorded at the end of the depolarizing pulse. Data points for the effect of increasing concentrations of CAF603 on the L-type Ca^{2+} currents were fitted to a simple adsorption isotherm or

'rectangular hyperbola', in order to calculate an apparent K_d for the inhibition of the Ca^{2+} currents.

2.3. Drugs

Stock solutions of CAF603 (isolated from strains of *T. virens*, as previously described by Ondeyka et al., 1995) were prepared at 100 mM in dimethylsulfoxide (DMSO), and were diluted appropriately in the experimental salines to obtain the required final concentration of CAF603. Iberiotoxin, purified from the scorpion *Buthus tamulus* (Galvez et al., 1990) was used in some experiments to block BK_{Ca} channels. Glyburide, atropine and tetrodotoxin were purchased from Sigma (St. Louis, MO, USA). All other reagents were analytical grade.

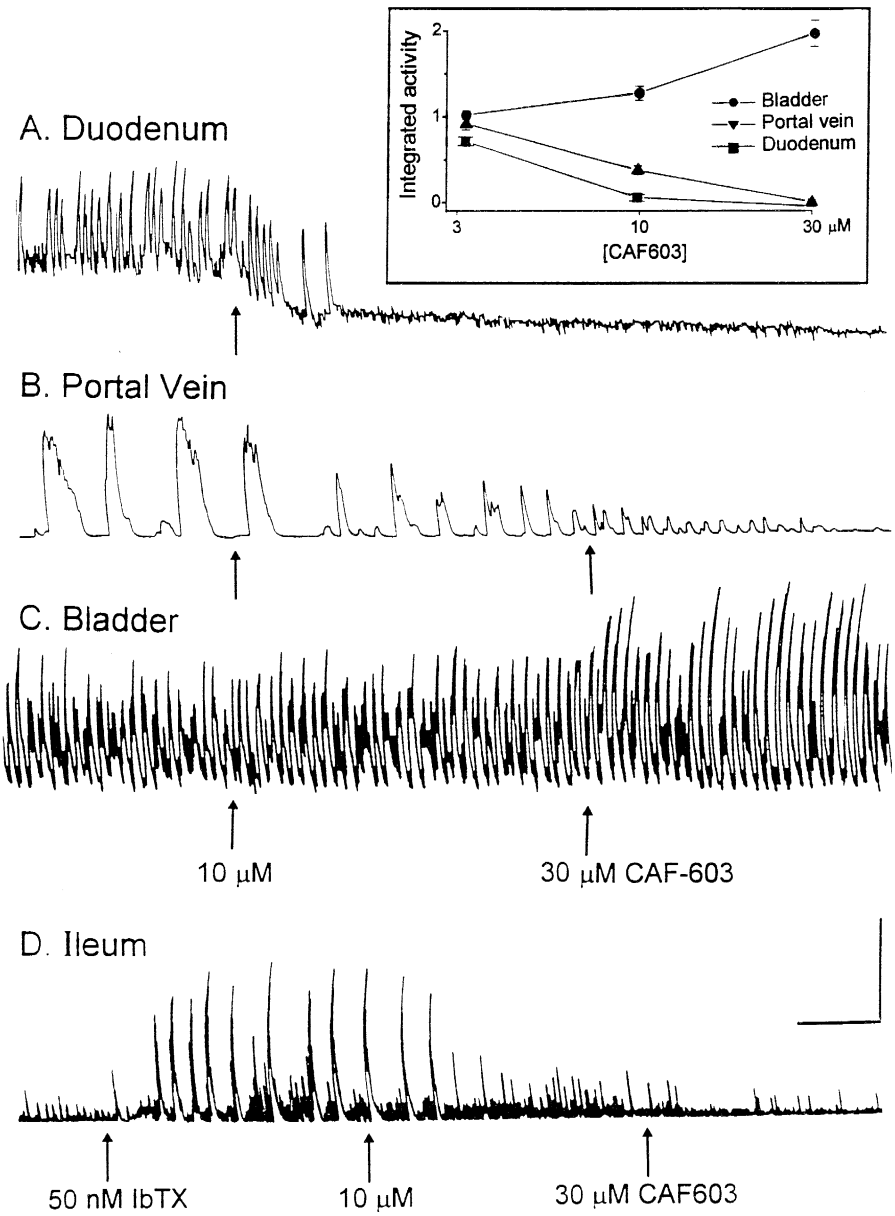

Fig. 2. Effect of CAF603 on the spontaneous motility of guinea-pig smooth muscles. (A–C) Isometric tension recordings from strips of duodenum, portal vein and detrusor muscle from the urinary bladder bathed in KHB, before and during exposure to 10 and 30 μM CAF603. (D) Isometric tension recordings from a strip of guinea-pig ileum, bathed in KHB, before and during exposure to iberiotoxin (50 nM) and to iberiotoxin plus 10 and 30 μM CAF603. Inset: Integrated mechanical activity data from portal veins ($n = 5$), and strips of duodenum (4) and urinary bladder (6), exposed for successive periods of 20 min to increasing concentrations of CAF603 (3–30 μM). The integrated activity measured between 15–20 min of exposure to each drug concentration was used to construct the plot. Data (mean ± S.E.M.) are expressed relative to the basal integrated activity, recorded in the 10 min period preceding exposure to the lowest concentration of CAF603. * $P < 0.05$, for the relative decrease (portal vein and duodenum) or increase (bladder) in spontaneous activity induced by CAF603. Calibration bars: horizontal, 5 min; vertical, 0.5 g (A, B), 1.0 g (C), 2.0 g (D).

2.4. Statistical analysis

Each experimental protocol was repeated at least 4 times. Pooled data from identical experiments are plotted as means ± S.E.M. Student's *t*-test was used for statistical analysis of the data. The significance level was set at $P < 0.05$.

3. Results

3.1. Effects of CAF603 on smooth muscle motility and tonus

The effects of CAF603 (1–100 μM) on the spontaneous motility of guinea-pig smooth muscle were tissue-selective. Concentration-dependent inhibition was observed in the portal vein, and in the ileum or duodenum strips, whereas the opposite effect, i.e., increased myogenic activity, occurred in detrusor muscle.

Representative tension recordings from portal vein, duodenum and detrusor muscle strips obtained from the same animal are shown in Fig. 2A–C. Tension data from 4–6 similar experiments were integrated in order to quantify the effects of CAF603 on these spontaneously active preparations, and results are plotted in Fig. 2 (inset). Duodenum strips showed greater sensitivity than portal vein strips to the relaxing effect of CAF603: thus, 10 μM CAF603 abolished the spontaneous motility of the duodenum, whereas 30 μM CAF603 were required for the same effect in the portal vein. At both these concentrations, CAF603 significantly increased the integrated contractility of detrusor muscle. This stimulatory effect on detrusor muscle was not reversed by either atropine (1 μM) or tetrodotoxin (10 μM; not shown).

3.2. Pharmacological interaction of CAF603 with iberiotoxin and glyburide

To investigate whether the relaxing effects of CAF603 on smooth muscle motility are related to modulation of the BK_{Ca} channel (see Section 1), iberiotoxin, a selective blocker of this channel, was used. The experiments were performed in ileum strips, which display marked increases in motility when exposed to iberiotoxin (50 nM), as shown in Fig. 2D. Addition of CAF603 (30 μM) to the medium reversed the stimulatory effect of iberiotoxin, reducing the spontaneous motility of the ileum strip. The fact that the relaxing effects of CAF603 persisted after blockade of the BK_{Ca} channels with iberiotoxin suggests to us that these channels do not mediate the CAF603-induced inhibition of smooth muscle motility. A possible role of ATP-dependent K channels as mediators of the relaxing effects of CAF603 was also discarded, since glyburide (10 μM), a selective blocker of these channels, failed to reverse the CAF603-induced inhibition of spontaneous motility in portal vein, duodenum or ileum strips (not shown).

3.3. CAF603 displays Ca^{2+}-entry blocker properties

The possibility that Ca^{2+}, rather than K^+ channels, mediate the inhibitory effect of CAF603 on smooth muscle motility, was investigated using a classical pharmacological paradigm for Ca^{2+}-entry blockade, i.e., Ca^{2+}-induced tension in depolarized guinea-pig ileum strips (Section 2). Two experimental protocols were used: In one, increasing concentrations of CAF603 were applied to preparations which were pre-contracted by addition of 1 mM $CaCl_2$ to the bathing medium. As shown in Fig. 3A, CAF603 caused a dose-dependent relaxation of the Ca^{2+}-induced tension. In the second protocol, concentration–response curves for $CaCl_2$ were constructed in the absence and in the presence of increasing concentrations of CAF603. The data (Fig. 3B) show that CAF603 displaced the tension/Ca^{2+} relationship to the right along the Ca^{2+} concentration axis. The drug concentration required for 50% inhibition of the tension induced by 3 mM $CaCl_2$ was estimated (data fitting to a single exponential) to be 22 μM. The effects of CAF603 in this experimental paradigm are similar to those

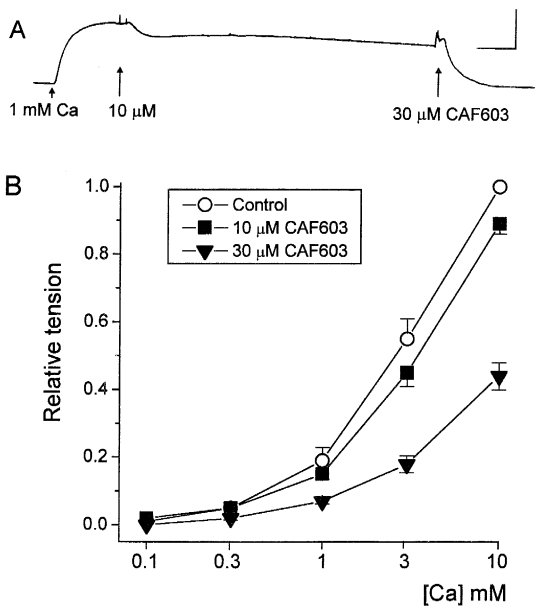

Fig. 3. Effect of CAF603 on the Ca^{2+}-induced tension in depolarized guinea-pig ileum strips. (A) Isometric tension recordings from a strip pre-equilibrated (30 min) with a nominally Ca^{2+}-free depolarizing solution, containing 43 mM KCl. Tension was induced by addition of 1.0 mM $CaCl_2$ to the depolarizing solution, to elicit a sustained, sub-maximal tension. CAF603 (10 and 30 μM) was added to the medium where indicated, and caused a concentration-dependent relaxation. Calibration bars: horizontal, 2 min; vertical, 0.5 g. In (B) the muscle strips were equilibrated with a nominally Ca^{2+}-free depolarizing (43 mM KCl) solution, and increasing concentrations of $CaCl_2$ were added to this solution in order to construct the tension/$[Ca^{2+}]$ curves shown in the plot. Between successive tests (control, 10 and 30 μM CAF603), the strips were soaked for 20 min in Ca^{2+}-free depolarizing solution. Data (mean ± S.E.M.) are expressed relative to the control tension elicited by 10 mM $[Ca^{2+}]$.

of standard Ca^{2+}-entry blockers, such as verapamil (see Section 4), and provide a possible mechanism for the inhibitory effect of CAF603 on the myogenic activity of guinea-pig ileum, duodenum and portal vein.

There is evidence (Suarez-Kurtz, 1993) that the myogenic activity of these tissues is abolished by verapamil concentrations, which have minimal or no effect on the spontaneous motility of guinea-pig detrusor muscle. Nevertheless, verapamil and other blockers of L-type Ca^{2+} channels, are effective blockers of K^+-induced contractions of detrusor muscle (Mostwin, 1985; Youssif et al., 1985). These observations prompted us to examine the effects of CAF603 on detrusor strips equilibrated with depolarizing salines containing 43 mM (Fig. 4A) or 25 mM KCl (Fig. 4B, C). Both these high-KCl solutions elicited biphasic contractions, with an initial transient contraction followed by a sustained tonic component. The spontaneous motility of detrusor muscle was abolished during the tonic phase in strips equilibrated with 43 mM KCl, but was enhanced in strips bathed in 25 mM KCl. Since the tonic component of the KCl-induced tension is more sensitive to Ca^{2+}-entry blockers (Youssif et al., 1985), CAF603 was applied after 15 min of exposure to the high-KCl salines. CAF603 (30 μM) caused a progressive decline of the tonus towards the basal level (Fig. 4A and B), and reduced the spontaneous motility of strips bathed in 25 mM KCl (Fig. 4B, C).

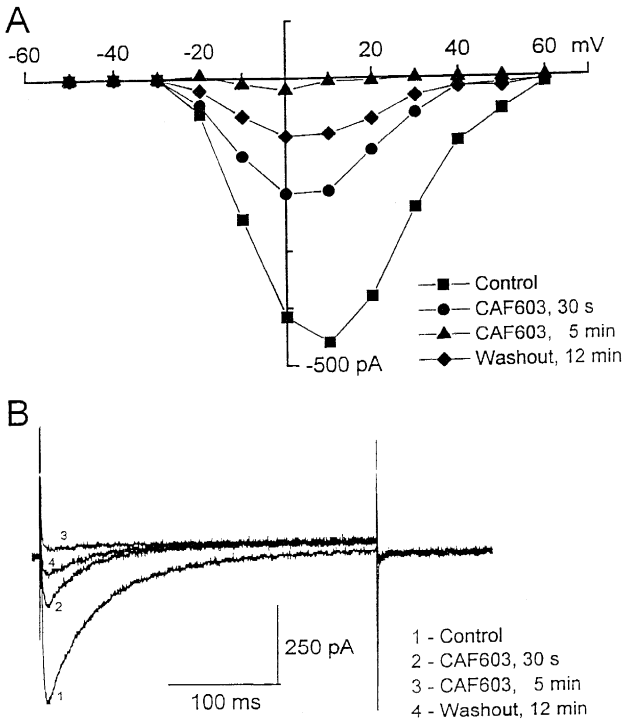

Fig. 5. Effect of CAF603 on the slow inward current in isolated myocytes from rabbit heart. The plot (A) shows the peak I_{si} at the various test pulses, applied from a holding potential of −40 mV, before (control, squares), after 30 s (circles) or 5 min (triangles) perfusion with 100 μM CAF603, and after 12 min of drug-free washout (diamonds). (B) shows current versus time records obtained at +10 mV, before, during and after the exposure to 100 μM CAF603, in the same experiment as in the plot.

3.4. Effects of CAF603 on L-type Ca^{2+} channel currents

Patch-clamp recordings of the slow inward current in rabbit myocytes provided direct evidence for a Ca^{2+}-entry blocking effect of CAF603. The experimental protocol was designed to eliminate both the fast Na^+ and the T-type Ca^{2+} currents (−40 mV holding potential), and all K^+ currents (Cs^+ and tetraethylammonium in the intracellular micropipette solution). The inward current (I_{si}) recorded during depolarizing pulses under these conditions corresponds to Ca^{2+} flowing through L-type channels. Fig. 5A shows *I–V* plots of peak I_{si} versus clamp potential, recorded in normal Tyrode's solution (control), during exposure to 100 μM CAF603, and after 12 min of drug washout. CAF603 induced a time-dependent reduction of

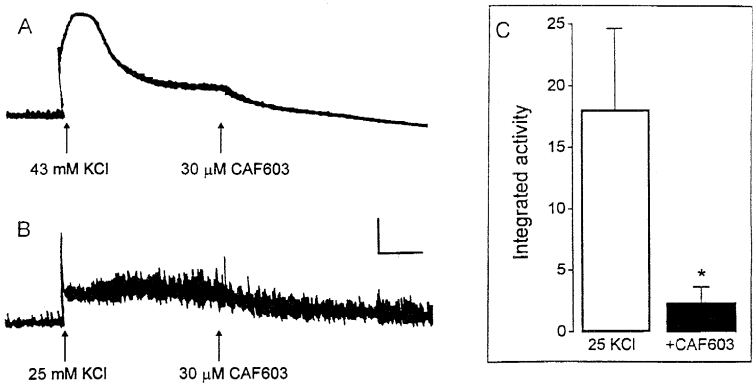

Fig. 4. Effect of CAF603 on the contractility of guinea-pig detrusor muscle bathed in depolarizing, high-KCl solutions. (A and B) Isometric tension recording from two muscle strips before and during exposure to iso-osmotic salines containing either 43 mM KCl (A) or 25 mM KCl (B), and subsequently treated with 30 μM CAF603. Calibration bars: horizontal, 5 min; vertical, 2 g. (C) Integrated mechanical activity data (means ± S.E.M) from 5 strips studied with the protocol depicted in (B). The bars correspond to the relative integrated mechanical activity (basal activity in control saline = 1) of strips equilibrated with the 25 mM KCl solution (left bar) and after addition of 30 μM CAF603 to this saline (10–15 min; right bar). * $p < 0.05$ for the difference in integrated activity before and after exposure to CAF603.

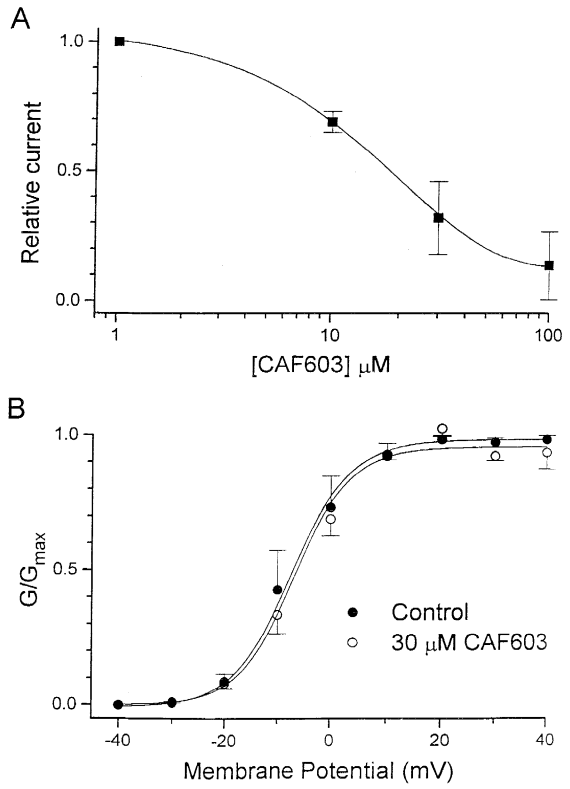

Fig. 6. (A) Concentration–response curve for the CAF603-induced inhibition of the L-type Ca^{2+} current in rabbit myocytes. Data points from 7 experiments were used to construct the plot. Relative current refers to the peak I_{si} at +10 mV, normalized to the control (drug-free) value. Drug effects were measured after 5–8 min perfusion with the concentration of CAF603 indicated in the abscissae. An apparent K_d of 19 μM for the Ca^{2+}-entry blocking effect of CAF603 was obtained by fitting the data points (solid line) to a simple adsorption isotherm. (B) Data from 4 cells were used to construct a plot of the normalized L-type Ca^{2+} channel conductance (G/G_{max}) vs. test potentials, before (control) and after 5–8 min exposure to 30 μM CAF603. In both conditions, G_{max} was observed at +20 mV.

peak I_{si}, which reversed partially upon return to Tyrode's solution. It is possible that L-type current rundown contributes, at least in part, to the incomplete recovery of the current amplitudes. The current traces reproduced in Fig. 5B show that CAF603 did not affect the time to peak of the maximum I_{si}, recorded at +10 mV.

Pooled data from seven experiments were used to construct the concentration–effect curve for the CAF603-induced blockade of the I_{si} in cardiac myocytes (Fig. 6A). An apparent K_d of 19 μM for the Ca^{2+}-entry blocking effect of CAF603 was obtained by fitting the data points to a simple adsorption isotherm. In Fig. 6B, the L-type Ca^{2+} channel conductance was plotted against the test potentials. The superposition of the data points measured in the absence or in the presence of CAF603 (30 μM) at each test potential indicates that the drug-induced blockage of I_{si} is not voltage-dependent, i.e., CAF603 does not affect the voltage dependence of the L-type Ca^{2+} current.

4. Discussion

CAF603 was discovered in natural products screening of fermentation extracts, using [^{125}I]charybdotoxin binding to BK_{Ca} channels in aortic sarcolemmal membranes. CAF603 was an allosteric inhibitor of charybdotoxin binding, causing 50% inhibition at 200 nM test compound. CAF603 had no effect on [^{125}I]charybdotoxin binding to voltage-dependent K^+ channels in rat brain synaptic plasma membranes. Despite its potency in modulating [^{125}I]charybdotoxin binding to BK_{Ca} channels, CAF603 at 10 μM had no consistent effect on the BK_{Ca} channel currents in lipid bilayer experiments after reconstitution of the aortic smooth muscle channel (Lee et al., 1995). Nevertheless, synthetic analogues of CAF603 displayed opposite effects on BK_{Ca} channel currents in membrane patches and in lipid bilayers. Thus, 14-hydroxy CAF603 oleate (10 μM) increased channel open probability, whereas application of 10 μM of either CAF603 oleate or CAF603 linoleate to the intracellular side of the BK_{Ca} channel decreased open channel probability (Lee et al., 1995). Although BK_{Ca} channel blockade might contribute to the stimulatory effect of CAF603 on the contractility of guinea-pig urinary bladder (see below), the predominant inhibitory effect of this compound on smooth muscle motility cannot be ascribed to activation of the BK_{Ca} channel. Two experimental observations strongly support this latter conclusion. First, iberiotoxin, a potent and selective blocker of smooth muscle BK_{Ca} channels (reviewed by Garcia et al., 1995) did not oppose the inhibitory effect of CAF603 on the spontaneous motility of guinea-pig ileum. Second, CAF603 inhibited the Ca^{2+}-induced tension in depolarized ileum strips and reduced the spontaneous motility and tonus of detrusor muscle equilibrated with saline solutions containing 25 or 43 mM KCl.

The data obtained in depolarized guinea-pig ileum provides indirect evidence for Ca^{2+}-entry blockade by CAF603. Indeed, this experimental paradigm is often used for screening for blockers of L-type Ca^{2+} channels in smooth muscle, and the ability of CAF603 to displace to the right the tension/pCa^{2+} relationship is mirrored by standard Ca^{2+}-entry blockers such as verapamil or the dihydropyridines (Triggle, 1981). Blockade of L-type Ca^{2+} channels provides a mechanism for the relaxing effect of CAF603 on ileum strips and other smooth muscle tissues examined, such as the guinea-pig portal vein, duodenum and depolarized detrusor muscle. It is significant that CAF603 did not inhibit the myogenic activity of detrusor muscle bathed in KHB; under these conditions, this preparation is relatively insensitive to L-type Ca^{2+}-entry blockers, such as verapamil (Suarez-Kurtz, 1993). The stimulatory effect of CAF603 in the detrusor muscle bathed in KHB will be dealt with at a later point of this discussion.

Patch-clamp experiments provided direct evidence for L-type Ca^{2+} channel blockade by CAF603. Thus, the I_{si} in rabbit ventricular myocytes was reduced in a dose-de-

pendent manner by CAF603, with an apparent K_d of 19 μM. This concentration is similar to that (22 μM) required for 50% inhibition of sub-maximal Ca^{2+}-induced tension in guinea-pig ileum. Considering the vastly different experimental conditions in which these values were obtained, their similarity strongly supports a common mechanism, namely blockade of L-type Ca^{2+} channels.

In contrast to its smooth muscle relaxant effects, which we ascribe to Ca^{2+}-entry blockade, CAF603 stimulated the spontaneous motility of detrusor muscle bathed in KHB. This effect must involve a different mechanism. Since neither atropine nor tetrodoxin reversed the stimulatory effect of CAF603 on detrusor muscle, it appears that this effect is not mediated by activation of muscarinic receptors or by the release of neurotransmitters. Inhibition of the BK_{Ca} channel could provide an explanation for the CAF603-induced stimulation of detrusor muscle contractility. This interpretation is consistent with the ability of CAF603 to allosterically modulate [^{125}I]charybdotoxin binding to the BK_{Ca} channel in smooth muscle membranes (Lee et al., 1995). Previous studies (Suarez-Kurtz et al., 1991; DeFarias et al., 1996) have shown that the spontaneous motility of guinea-pig detrusor is exquisitely sensitive to both peptidyl (charybdotoxin and iberiotoxin) and non-peptidyl (paspalitrem and paxilline) blockers of BK_{Ca} channels. Although CAF603, at 10 μM, had no consistent effect on BK_{Ca} currents recorded by Lee et al. (1995), it is possible that higher concentrations of this compound, such as those required to stimulate the contractility of detrusor muscle, block BK_{Ca} channels.

In conclusion, we have shown that CAF603, a carotane sesquiterpene isolated from the fungus *Trichoderma virens*, and identified in a [^{125}I]charybdotoxin binding assay as a modulator of the BK_{Ca} channel, is also a L-type Ca^{2+} channel entry blocker. The latter property underlies the drug-induced relaxation of various smooth muscle tissues, whereas the blockade of BK_{Ca} channels is thought to account for the increased contractility of detrusor muscle bathed in KHB.

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