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EFFECTS OF FELODIPINE (A DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKER) AND ANALOGUES ON CALMODULIN-DEPENDENT ENZYMES

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Abstract—We have examined the effects on the activities of three calmodulin-dependent enzymes (cAMP phosphodiesterase, caldesmon kinase and myosin light chain kinase) of the dihydropyridine Ca^{2+} channel blocker felodipine and three analogues (*p*-chloro, oxidized and *t*-butyl) exhibiting different pharmacological potencies. The cAMP phosphodiesterase was inhibited completely by felodipine and the *p*-chloro analogue with IC_{50} values of 3.7 and 1.5 μM respectively. The oxidized and *t*-butyl analogues were relatively ineffective in inhibiting cAMP phosphodiesterase. Felodipine and the *p*-chloro analogue inhibited the basal (Ca^{2+}/calmodulin-independent) activity of cAMP phosphodiesterase as well as the calmodulin-stimulated activity. Calmodulin was relatively ineffective in preventing inhibition of cAMP phosphodiesterase by felodipine and the *p*-chloro analogue. These observations suggest that felodipine may act directly on the phosphodiesterase as well as through calmodulin. Felodipine and the *p*-chloro analogue inhibited Ca^{2+}/calmodulin-dependent caldesmon kinase with similar potencies (IC_{50} = 17.4 μM), whereas the oxidized and *t*-butyl analogues caused no inhibition. Similarly, felodipine and the *p*-chloro analogue inhibited myosin light chain kinase activity whether the isolated 20 kD light chain (IC_{50} = 12.6 μM) or intact myosin (IC_{50} = 11.0 μM) was used as substrate. Inhibition in each case was prevented by excess calmodulin. The oxidized and *t*-butyl derivatives caused little or no inhibition. Finally, the effects of felodipine and the three analogues on two processes which are dependent on myosin phosphorylation were examined, namely the actin-activated Mg^{2+}-ATPase activity of myosin and the assembly of myosin filaments. Felodipine and the *p*-chloro analogue inhibited the actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin (IC_{50} = 25.1 μM). The oxidized and *t*-butyl analogues exhibited no inhibition. Similarly, felodipine and the *p*-chloro analogue blocked myosin filament assembly induced by low concentrations of calmodulin, whereas the oxidized and *t*-butyl analogues did not. Again, inhibition of the actin-activated myosin Mg^{2+}-ATPase and myosin filament assembly by felodipine and the *p*-chloro analogue could be reversed by raising the calmodulin concentration. These observations suggest that some of the pharmacological actions of felodipine on smooth muscle may involve inhibition of calmodulin-dependent enzymes which are functionally involved in the regulation of smooth muscle contraction.

Felodipine [4-(2,3-dichlorophenyl)-1,4-dihydropyridine-2,6-dimethyl 3,5-dicarboxylic 3-ethyl ester and 5-methyl ester] is a potent vasodilating agent of the dihydropyridine calcium antagonist group of compounds [1] with selectivity for arterial resistance vessels [2]. The inhibition of vascular smooth muscle contraction by dihydropyridine Ca^{2+} channel blockers (e.g. nifedipine) is thought to be due primarily to its action on voltage-operated calcium channels, blocking Ca^{2+} entry into the cell [3]. However, additional (intracellular) sites of action of felodipine and related dihydropyridine calcium channel blockers have been suggested [1]. In support of such a notion, felodipine interacts *in vitro* with calmodulin in a Ca^{2+}-dependent manner [4–6], and recent photoaffinity labeling studies have demonstrated the specific binding of [^{3}H]felodipine to a soluble protein of porcine mesenteric arterial smooth muscle of subunit M_r 62 kD [7].

Earlier studies have demonstrated an inhibition of two calmodulin-dependent enzymes, cAMP phosphodiesterase [1, 8–10] and myosin light chain kinase [1, 11, 12], by felodipine. Inhibition of these enzymes was observed with K_i values in the 1–10 μM range, compared with half-maximal inhibition of contraction of vascular smooth muscle strips at felodipine concentrations in the nanomolar range [4, 12]. This apparent discrepancy does not necessarily mean that the mechanism of action of felodipine does not involve its interaction with calmodulin, since Ca^{2+} antagonists like felodipine can accumulate in smooth muscle cells [1, 13]. We have pursued this problem by investigating the effects of felodipine and three analogues exhibiting different pharmacological potencies on the activities of three calmodulin-dependent enzymes which are involved in the regulation of smooth muscle contraction: cAMP phosphodiesterase, caldesmon kinase and myosin light chain kinase. In addition, the effects of the same drugs on two processes that are regulated by myosin phosphorylation, namely actin-activated myosin Mg^{2+}-ATPase activity and myosin filament assembly, were examined.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (10–40 Ci/mmol) was purchased from Amersham (Oakville, Ontario,

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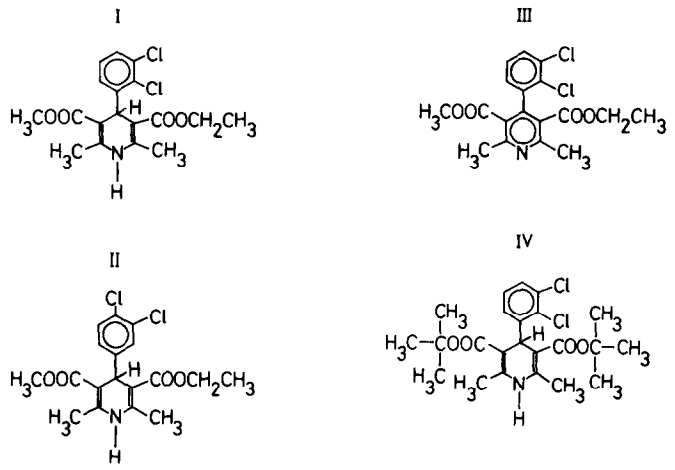

Fig. 1. Felodipine and its analogues used in this study. (I) felodipine; (II) *p*-chloro analogue; (III) oxidized analogue; and (IV) *t*-butyl analogue.

Canada). M_r markers for SDS*/polyacrylamide gel electrophoresis and 5′-nucleotidase were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Felodipine and its analogues (Fig. 1) were supplied by Hässle Pharmaceutical, Mölndal, Sweden. Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific Ltd. (Calgary, Alberta, Canada). Stock solutions of felodipine and its analogues (10 mM) were made up fresh at the start of each assay in absolute ethanol. The maximum final concentration of ethanol in the assay mixtures was 0.56% (v/v); this concentration had no effect on any of the activities measured.

Protein purifications. The following proteins were purified as previously described: calmodulin [14] and cAMP phosphodiesterase [15] from bovine brain, actin [16], myosin [17], the isolated 20 kD light chain of myosin [18], tropomyosin [19], myosin light chain kinase [20], a partially purified preparation of myosin light chain phosphatase [21], and caldesmon containing caldesmon kinase activity [20], all from chicken gizzard smooth muscle. The specific activity (expressed as μmol P_i incorporated per min per mg enzyme of the myosin light chain kinase preparation under optimal conditions was 13.2 using the isolated 20 kD light chain as substrate or 4.0 using intact myosin as substrate.

Enzyme assays. The results presented are representative of at least three sets of experiments using different protein preparations, unless otherwise indicated. Cyclic AMP phosphodiesterase activity was measured by incubation of the bovine brain enzyme, at a final concentration of 2.2 μg/ml, at 30° in 40 mM Tris–HCl, 40 mM imidazole–HCl (pH 7.5), 3 mM magnesium acetate, 0.11 mM $CaCl_2$ or 0.11 mM EGTA, 30 nM calmodulin, 0.28units/ml 5′-nucleotidase in the presence and absence of various concentrations of felodipine and its analogues. Reactions (total volume 4.95 ml) were initiated by the addition of cAMP to a final concentration of 1.2 mM. Samples (0.45 ml) of reaction mixtures were withdrawn at 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 min for quantification of cAMP hydrolysis by a coupled enzymatic assay as previously described [22]. Rates of cAMP hydrolysis were calculated from the linear time–course assays and a standard curve relating $A_{660\,nm}$ to μmol P_i, and are expressed as μmol P_i released per min per mg phosphodiesterase.

Caldesmon kinase activity was measured by incubation of the caldesmon/caldesmon kinase preparation, at a final concentration of 0.7 μM, at 30° in 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 1 mM EGTA, 1 nM calmodulin, 0.5 mM [γ-^{32}P]ATP (~9000 cpm/nmol) in the presence and absence of various concentrations of felodipine and its analogues. Reactions (total volume 1.5 ml) were initiated by the addition of [γ-^{32}P]ATP, and samples (0.15 ml) were withdrawn at 5, 10, 15, 20, 25, 30, 40, 50 and 60 min for quantification of [^{32}P]P_i incorporation as previously described [23]. The remainder of each reaction was quenched at 65 min by addition of an equal volume (0.15 ml) of SDS gel sample buffer [25 mM Tris–HCl (pH 6.8), 0.5% (w/v) SDS, 0.005% (w/v) Bromphenol Blue, 15% (v/v) glycerol, 0.5% 2-mercaptoethanol] and boiled. Samples (50 μl containing 2.5 μg caldesmon) were subjected to SDS/polyacrylamide-gradient slab-gel electrophoresis and autoradiography as described below.

Myosin light chain kinase activity was measured using either the isolated 20 kD light chain or intact myosin as substrate. In the former case, myosin light chain kinase was incubated, at a final concentration of 10 nM, at 30° in 25 mM Tris–HCl (pH 7.5), 60 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 1 mM EGTA, 0.5 nM calmodulin, 4.2 μM LC_{20}, 1 mM [γ-^{32}P]ATP (~7000 cpm/nmol) in the presence and absence of various concentrations of felodipine and its

* Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; LC_{20} and LC_{17}, the 20 kD and 17 kD light chains of myosin; and SDS, sodium dodecyl sulfate.

analogues. Reactions (total volume 1.6 ml) were initiated by the addition of [γ-^{32}P]ATP, and samples (0.2 ml) were withdrawn at 1, 2, 3, 4, 5, 6 and 7 min for quantification of [^{32}P]P_i incorporation as previously described [23]. When intact myosin was used as substrate, myosin light chain kinase was incubated, at a final concentration of 10 nM, at 30° in 25 mM Tris–HCl (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 1 mM EGTA, 1 nM calmodulin, 1 μM myosin, 1 mM [γ-^{32}P]ATP (~1500 cpm/nmol) in the presence and absence of various concentrations of felodipine and its analogues. Reactions (total volume 4.0 ml) were initiated by the addition of [γ-^{32}P]ATP, and samples (0.5 ml) were withdrawn at 1, 2, 3, 4, 5, 6 and 7 min for quantification of [^{32}P]P_i incorporation as previously described [23].

Actin-activated myosin Mg^{2+}-ATPase activities were measured at 30° under the following conditions: 25 mM Tris–HCl (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 1 mM EGTA, 1 nM calmodulin, 5.95 μM actin, 1 μM tropomyosin, 0.91 μM myosin, 10 nM myosin light chain kinase, 1 mM [γ-^{32}P]ATP (~3000 cpm/nmol) in the presence and absence of various concentrations of felodipine and its analogues. Reactions (total volume 4.0 ml) were initiated by the addition of [γ-^{32}P]ATP, and samples (0.5 ml) were withdrawn at 1, 2, 3, 4, 5, 6 and 7 min for quantification of [^{32}P]P_i released as previously described [24].

Myosin filament assembly. The reversible assembly of smooth muscle myosin into filaments was verified by incubating myosin (2.1 μM) at 21° in 25 mM Tris–HCl (pH 7.5), 0.15 M KCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.25 mM DTT, 38.4 nM myosin light chain kinase in a reaction volume of 1.0 ml. Under these conditions, myosin exists in filamentous form. Myosin filament assembly–disassembly was monitored by recording $A_{340\,nm}$ [25]. Filaments were disassembled by addition of 2.5 mM ATP and induced to reassemble by addition of $CaCl_2$ (0.1 mM excess over EGTA) and 1 μM calmodulin, whereupon myosin phosphorylation occurs. Subsequent addition of partially purified myosin light chain phosphatase (50 μl/ml) and EGTA (0.1 mM excess over Ca^{2+}) leads to filament disassembly. To examine the effects of felodipine and its analogues on myosin filament assembly–disassembly, myosin filaments were initially formed by incubating myosin (2.1 μM) at 21° in 25 mM Tris–HCl (pH 7.5), 0.15 M KCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.25 mM DTT, 20 nM myosin light chain kinase in a reaction volume of 1.0 ml. Filaments were disassembled by addition of 2.5 mM ATP. The effects of $CaCl_2$ (0.1 mM excess over EGTA) with either 5 nM calmodulin or 5 nM calmodulin plus 22.2 μM felodipine or analogue on myosin filament assembly were examined by recording $A_{340\,nm}$.

Protein concentrations. Protein concentrations were determined by the Coomassie Blue dye-binding assay [26] using dye reagent and γ-globulin standard purchased from the Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements using the following absorption coefficients: calmodulin, $A^{1\%}_{277\,nm}$ = 1.9 [27]; actin, $A^{1\%}_{290\,nm}$ = 6.5 [28]; myosin, $A^{1\%}_{280\,nm}$ = 5.6 [29].

Electrophoresis. Electrophoresis was performed in 7.5–20% polyacrylamide-gradient slab gels (1.5 mm thick), with a 5% acrylamide stacking gel, in the presence of 0.1% (w/v) SDS at 36 mA, employing the discontinuous buffer system of Laemmli [30]. Gels were stained in 45% (v/v) ethanol/10% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and diffusion-destained in 10% (v/v) acetic acid. Destained gels were sealed in plastic bags and autoradiographed using Kodak X-Omat AR film in Kodak X-Omat AR cassettes fitted with intensifying screens. Films were allowed to develop for 1 week or less at room temperature. Scanning densitometry of destained gels was carried out in an LKB model 2202 Ultroscan laser densitometer equipped with a Hewlett–Packard model 3390A integrator. Molecular weights were calculated from mobilities in SDS/7.5–20% polyacrylamide-gradient slab-gels by plotting log M_r versus log T%, where T = acrylamide concentration at a given point in the gel [31], using the following M_r markers: α-lactalbumin (14.2 kD), soybean trypsin inhibitor (20.1 kD), trypsinogen (24 kD), carbonic anhydrase (29 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), ovalbumin (45 kD), bovine serum albumin (66 kD), phosphorylase *b* (97.4 kD), β-galactosidase (116 kD), and myosin heavy chain

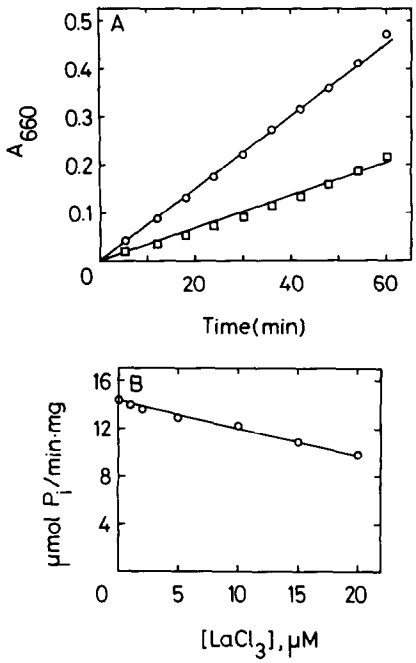

Fig. 2. Effect of La^{3+} on calmodulin-stimulated cAMP phosphodiesterase. (A) Phosphodiesterase activity was measured, as described in Materials and Methods, in the presence of Ca^{2+} and in the presence (□) and absence (○) of 20 μM $LaCl_3$. Samples (0.45 ml) of reaction mixtures were withdrawn at the indicated times for quantification of cAMP hydrolysis by a coupled enzymatic assay involving measurement of P_i released ($A_{660\,nm}$). (B) Assays similar to that shown in panel A were carried out at several concentrations of $LaCl_3$. From the linear time–course assays of cAMP hydrolysis, enzymatic rates were calculated and are plotted as a function of La^{3+} concentration.

(205 kD). Urea/polyacrylamide slab-gel electrophoresis in the presence of glycerol was carried out using the system of Perrie and Perry [32] as described by Kendrick-Jones *et al.* [25].

RESULTS

Cyclic AMP phosphodiesterase. We initiated this study by examining the effects of felodipine and its analogues on the activity of calmodulin-stimulated cAMP phosphodiesterase. It was decided to examine such effects in the presence and absence of La^{3+}, since Mills and Johnson [6] have shown that La^{3+} enhances the affinity of calmodulin for felodipine by approximately 20-fold, with half-maximal effect at 8 μM La^{3+}. It was therefore necessary initially to determine whether La^{3+} has any effect directly on the calmodulin-stimulated cAMP phosphodiesterase. Figure 2A indicates an inhibitory effect of 20 μM La^{3+} on the phosphodiesterase in a time–course assay. The dose–response curve in Fig. 2B indicates a linear relationship between phosphodiesterase activity and La^{3+} concentration. The mean percent inhibition (±SD) at 20 μM $LaCl_3$ was determined to be 37.4 ± 4.7% (N = 6). La^{3+} can substitute for Ca^{2+} in activating cAMP phosphodiesterase, although activation by La^{3+} is not as great as by Ca^{2+}: phosphodiesterase activity in the presence of 0.11 mM Ca^{2+} was 15.9 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ and in the presence of 20 μM La^{3+} was 13.7 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$. Chelation of metal ions with EGTA reduced the hydrolysis rate to 2.8 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$.

Subsequent experiments designed to investigate the effects of felodipine and its analogues on cAMP phosphodiesterase activity were carried out in the presence and absence of 20 μM $LaCl_3$.

The dose–response curves in Fig. 3 illustrate the effects of felodipine (A), and its *p*-chloro (B), oxidized (C) and *t*-butyl (D) analogues on the Ca^{2+}/calmodulin-dependent cAMP phosphodiesterase both in the presence and absence of La^{3+}. These data illustrate several points: (1) In the absence of La^{3+}, felodipine and the *p*-chloro analogue completely inhibited the phosphodiesterase, with IC_{50} values of 3.7 and 1.5 μM respectively. On the other hand, little inhibition by the oxidized and *t*-butyl analogues was observed until high concentrations (10^{-5}–10^{-4} M) were reached. (2) Similar results were obtained in the presence of La^{3+}, inhibition by felodipine and the *p*-chloro analogue occurring with IC_{50} values of 2.8 and 1.1 μM respectively. Little

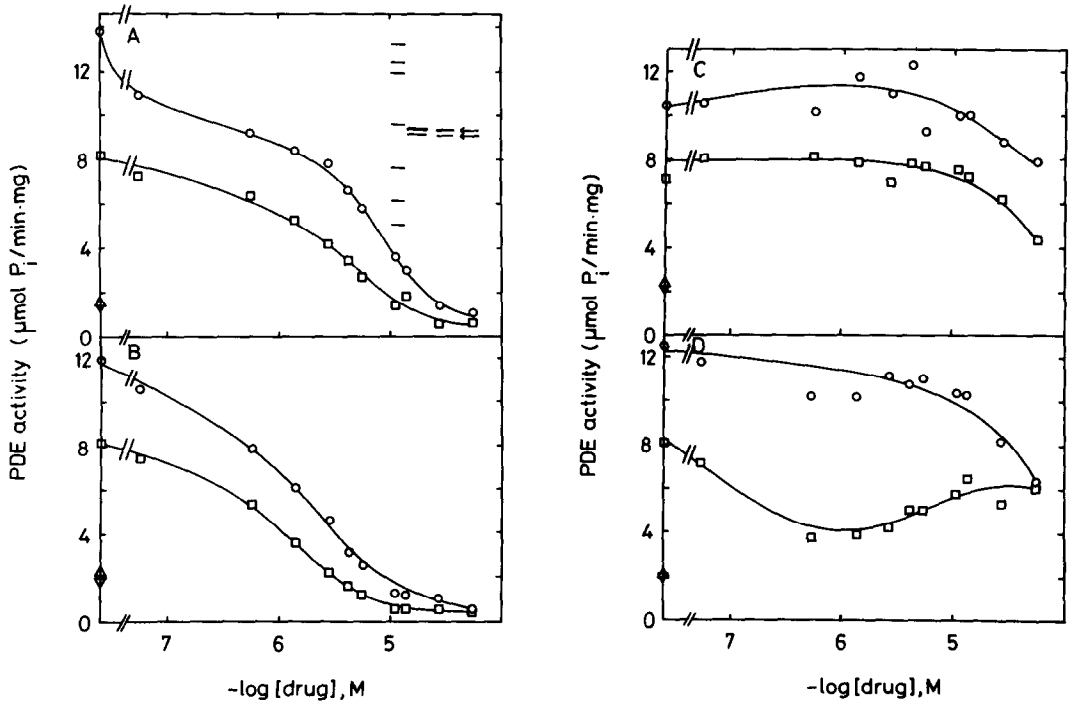

Fig. 3. Effects of felodipine and its analogues on cAMP phosphodiesterase activity in the presence and absence of La^{3+}. Phosphodiesterase activity was assayed, as described in Materials and Methods, in the presence of various concentrations of felodipine (A) or its analogues: *p*-chloro (B), oxidized (C) and *t*-butyl (D); and in the presence (□) and absence (○) of 20 μM $LaCl_3$. The basal rates of cAMP phosphodiesterase in the absence of Ca^{2+} and drug and in the presence (▽) and absence (△) of La^{3+} are also indicated. The gel inset in panel A shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of two phosphodiesterase preparations (10 μg protein/lane). Scanning densitometry demonstrated that the two phosphodiesterase isoenzymes (M_r 60 and 63 kD) [33], which are indicated by the arrows, constituted 56.7% of total protein in each preparation. The positions of M_r markers electrophoresed simultaneously are indicated by horizontal bars to the left of the gels. From top to bottom, their M_r values are 205, 116, 97.4, 66, 45, 36, and 29 kD.

Table 1. Effect of excess calmodulin on inhibition of cAMP phosphodiesterase by felodipine and its analogues

Drug	[Calmodulin]	cAMP phosphodiesterase activity* (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$) $-La^{3+}$	$+La^{3+}$
None	30 nM	11.91	7.80
	1 μM	11.93	9.18
Felodipine	30 nM	1.08	0.62
	1 μM	1.88	0.56
p-Chloro analogue	30 nM	0.58	0.42
	1 μM	0.83	1.24
Oxidized analogue	30 nM	7.84	4.34
	1 μM	8.86	8.94
t-Butyl analogue	30 nM	6.43	6.05
	1 μM	12.75	9.29

* cAMP phosphodiesterase activity was measured, as described in Materials and Methods, in the presence of the indicated drug (55.6 μM) at either 30 nM or 1 μM calmodulin in the presence and absence of 20 μM $LaCl_3$.

inhibition was observed by the oxidized derivative. In the case of the *t*-butyl analogue in the presence of La^{3+}, substantial inhibition was observed only at low drug concentrations. (3) Felodipine and the *p*-chloro analogue inhibited the basal activity (the activity observed in the absence of Ca^{2+}) of cAMP phosphodiesterase as well as the Ca^{2+}/calmodulin-stimulated activity. (4) La^{3+} did not inhibit the basal activity, but did cause partial inhibition of the Ca^{2+}/calmodulin-stimulated activity as seen in Fig. 2. (5) Inhibition by the highest concentrations of felodipine or *p*-chloro analogue used (5.56×10^{-5} M) could not be reversed by calmodulin (Table 1). However, the partial inhibition observed at high concentrations of the oxidized and *t*-butyl analogue was reversed by calmodulin, although only partially in the case of the oxidized derivative in the absence of La^{3+} (Table 1).

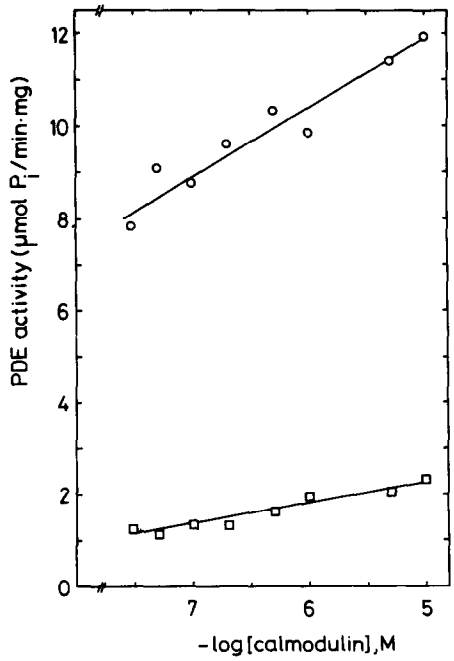

Fig. 4. Effect of increasing calmodulin concentrations on inhibition of cAMP phosphodiesterase activity by felodipine. Phosphodiesterase activity was assayed in the presence of Ca^{2+}, as described in Materials and Methods, at the indicated concentrations of calmodulin in the presence of either 2.78 μM (○) or 55.6 μM (□) felodipine. Phosphodiesterase activities in the absence of felodipine and in the presence of 30 nM calmodulin were 11.5 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ in the presence of Ca^{2+} and 2.0 μmol $P^i \cdot min^{-1} \cdot mg^{-1}$ in the absence of Ca^{2+}.

This last point was investigated in more detail by studying the effect of varying calmodulin concentration on cAMP phosphodiesterase activity in the presence of 55.6 μM felodipine (which gave almost complete inhibition at 30 nM calmodulin) and 2.78 μM felodipine (which gave approx. 32% inhibition at 30 nM calmodulin) (Fig. 4). Calmodulin induced complete reversal of inhibition at the low drug concentration, but only partial reversal at the high drug concentration.

The data in Fig. 3 indicated inhibition of the basal (Ca^{2+}-independent) activity of cAMP phosphodiesterase by felodipine and the *p*-chloro analogue. This conclusion was substantiated in a separate series of experiments using a preparation of cAMP phosphodiesterase that was partially proteolysed by an endogenous proteinase. Limited proteolysis of cAMP phosphodiesterase results in loss of the Ca^{2+}- and calmodulin-dependence of the enzymatic activity [34]. This preparation exhibited a specific activity of 13.0 μmol P_i released $\cdot$ min^{-1} $\cdot$ (mg enzyme)$^{-1}$ in the presence of Ca^{2+} and calmodulin, and 11.0 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ in the absence of Ca^{2+}. Felodipine and the *p*-chloro analogue (55.6 μM) inhibited this activity to 1.5 and 1.9 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$, respectively, whether measured in the presence or absence of Ca^{2+}/calmodulin.

Caldesmon kinase. The effects of felodipine and the three analogues on Ca^{2+}/calmodulin-dependent caldesmon kinase activity are shown in Fig. 5. Felodipine and the *p*-chloro analogue inhibited this kinase with $IC_{50} = 17.4$ μM, whereas no inhibition was observed at concentrations of the oxidized or *t*-butyl analogue as high as 55.6 μM. The gel inset (Fig. 5, lane A) shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of the caldesmon/caldesmon kinase preparation. The arrow depicts the caldesmon polypeptide (M_r 141 kD). The major lower M_r polypeptides represent proteolytic fragments of caldesmon [35]. The autoradiograms (Fig. 5, lanes B–G) illustrate the phosphorylation of caldesmon in a Ca^{2+}-dependent manner and its inhibition by felodipine and the *p*-chloro analogue, but not by the oxidized or *t*-butyl analogue. Inhibition by felodipine or the *p*-chloro analogue was overcome completely by raising the calmodulin concentration from 1 nM to 1 μM (data not shown).

Myosin light chain kinase. The effects of felodipine and its analogues on Ca^{2+}/calmodulin-dependent myosin light chain kinase activity were examined initially using the isolated 20 kD light chain of smooth

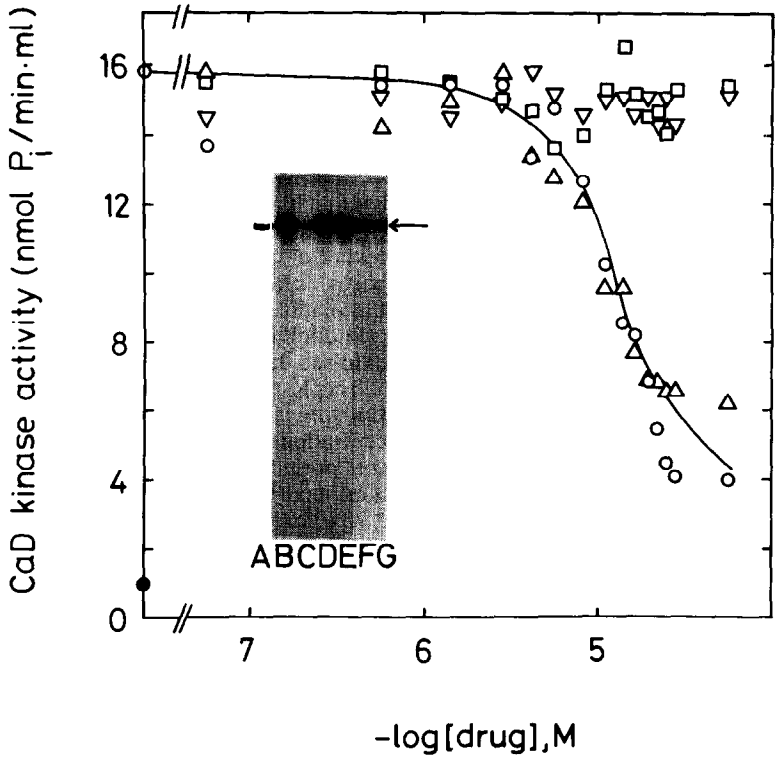

Fig. 5. Effects of felodipine and its analogues on Ca^{2+}/calmodulin-dependent caldesmon kinase activity. Caldesmon kinase activity was measured, as described in Materials and Methods, in the presence of various concentrations of felodipine (○) or its analogues: *p*-chloro (△), oxidized (▽) and *t*-butyl (□). The low basal activity of the enzyme in the absence of Ca^{2+} is indicated (●). The inset shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of the caldesmon/caldesmon kinase preparation (lane A) and autoradiograms (lanes B–G) of the following reaction mixtures: $+Ca^{2+}$, no drug (B); $-Ca^{2+}$, no drug (C); $+Ca^{2+}$ + *t*-butyl analogue (D); $+Ca^{2+}$ + oxidized analogue (E); $+Ca^{2+}$ + felodipine (F); and $+Ca^{2+}$ + *p*-chloro analogue (G). Where present, the drug concentration in these samples was 55.6 μM, i.e. the highest concentration utilized. The arrow indicates the position of the caldesmon band (M_r 141 kD).

muscle myosin (LC_{20}) as substrate. Both substrate and enzyme were shown to be electrophoretically homogeneous. Stoichiometric phosphorylation of LC_{20} (0.95 mol P_i/mol LC_{20}) occurred in the presence, but not in the absence (0.03 mol P_i/mol LC_{20}), of Ca^{2+} when LC_{20} (4.2 μM) was incubated with 73.5 nM myosin light chain kinase under phosphorylation conditions as described in Materials and Methods.

Figure 6 illustrates the effects of felodipine and the three analogues on myosin light chain kinase activity using the isolated LC_{20} as substrate. Felodipine and the *p*-chloro analogue inhibited myosin light chain kinase with IC_{50} = 12.6 μM. No significant inhibition was observed at concentrations of the oxidized and *t*-butyl analogues up to 55.6 μM. Inhibition by felodipine or the *p*-chloro analogue was overcome completely by raising the calmodulin concentration from 0.5 nM to 0.1 μM (data not shown).

Similar results were obtained if intact myosin rather than the isolated LC_{20} was used as substrate for the myosin light chain kinase (Fig. 7). Felodipine and the *p*-chloro analogue inhibited myosin light chain kinase activity with IC_{50} = 11.0 μM. No significant inhibition by the oxidized and *t*-butyl analogues was observed, except at the highest concentrations of the *t*-butyl analogue used, where partial inhibition was observed. Inhibition by felodipine or the *p*-chloro analogue was overcome completely by raising the calmodulin concentration from 1 nM to 0.1 μM (data not shown).

Actin-activated myosin Mg^{2+}-ATPase. The effects of felodipine and its analogues on the actin-activated Mg^{2+}-ATPase of smooth muscle myosin were examined in a system reconstituted from purified actin, myosin, tropomyosin, calmodulin and myosin light chain kinase. In an initial series of experiments (Table 2), felodipine and the *p*-chloro analogue induced substantial inhibition of the actin-activated myosin Mg^{2+}-ATPase (70.2 and 73.3% inhibition respectively) at 1 nM calmodulin. This correlated with comparable inhibition (76.5% in each case) of myosin phosphorylation. On the other hand, the oxidized and *t*-butyl analogues did not inhibit either myosin phosphorylation or the actin-activated myosin Mg^{2+}-ATPase under identical conditions. The inhibition by felodipine and the *p*-chloro analogue of both myosin phosphorylation and actin-activated myosin Mg^{2+}-ATPase activity was prevented by increasing the calmodulin concentration to 1 μM (Table 2).

Figure 8 shows the effects of increasing con-

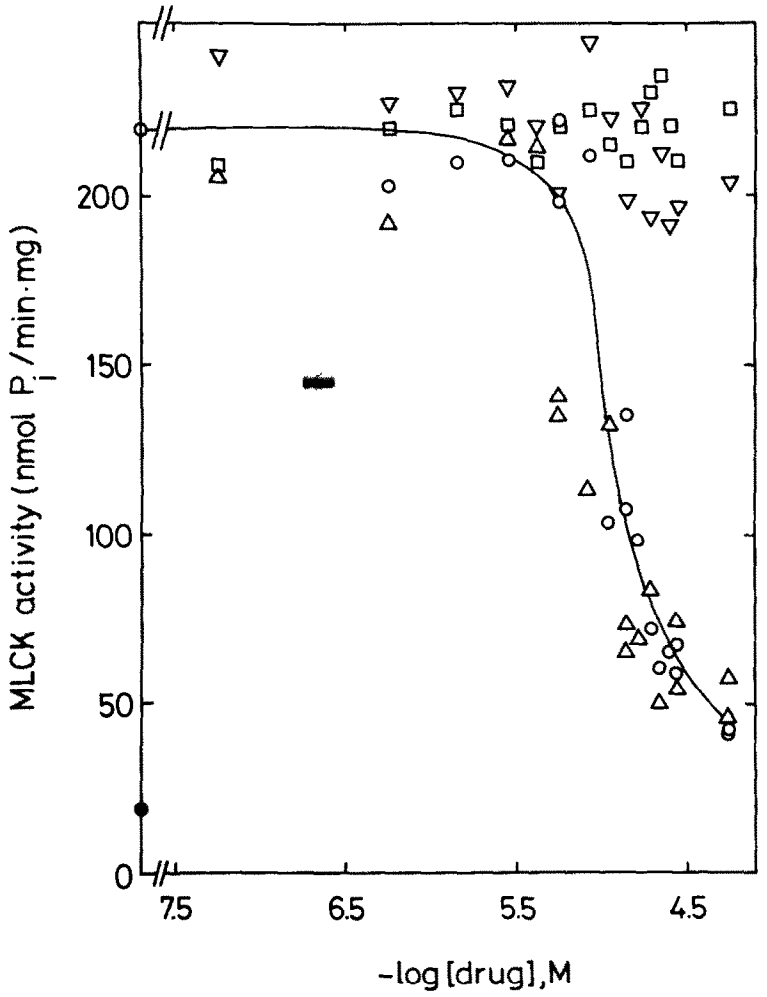

Fig. 6. Effects of felodipine and its analogues on myosin light chain kinase activity using LC_{20} as substrate. Myosin light chain kinase activity was measured, as described in Materials and Methods, in the presence of various concentrations of felodipine (○) or its analogues: *p*-chloro (△), oxidized (▽) and *t*-butyl (□), using the isolated myosin LC_{20} as substrate. The low level of enzymatic activity in the absence of Ca^{2+} is indicated (●). The inset shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of the myosin light chain kinase preparation (10 μg; *M*, 136 kD).

Table 2. Effect of felodipine and its analogues on actin-activated myosin Mg^{2+}-ATPase activity and myosin phosphorylation

Conditions		ATPase rate*	Phosphorylation level†
[CaM]	Drug	[nmol $P_i \cdot min^{-1} \cdot$ (mg myosin)$^{-1}$]	[mol P_i(mol myosin)$^{-1}$]
1 nM	None	90.2	1.7
1 nM	Felodipine	26.9	0.4
1 nM	*p*-Chloro analogue	24.1	0.4
1 nM	Oxidized analogue	93.1	1.5
1 nM	*t*-Butyl analogue	94.7	1.7
1 μM	None	90.3	1.7
1 μM	Felodipine	93.9	1.6
1 μM	*p*-Chloro analogue	96.0	2.0
1 μM	Oxidized analogue	104.6	1.7
1 μM	*t*-Butyl analogue	105.5	1.7

Values represent means of duplicate determinations.
* Measured under conditions described in Materials and Methods.
† Measured after 4-min incubation.

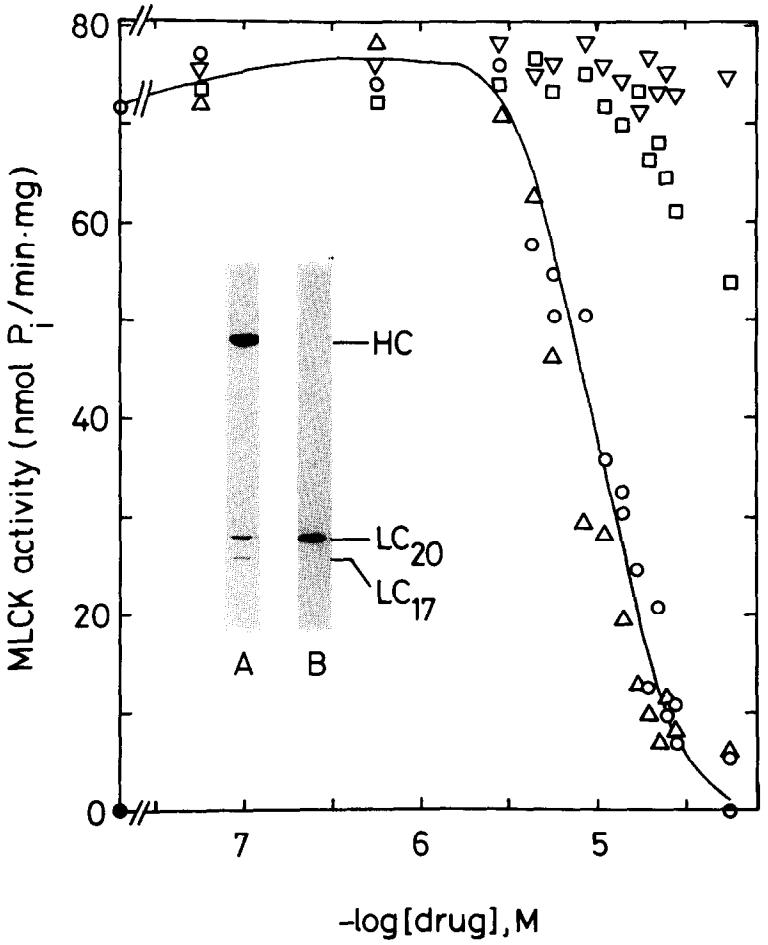

Fig. 7. Effects of felodipine and its analogues on myosin light chain kinase activity using intact myosin as substrate. Myosin light chain kinase activity was measured, as described in Materials and Methods, in the presence of various concentrations of felodipine (○) or its analogues: *p*-chloro (△), oxidized (▽) and *t*-butyl (□), using intact myosin as substrate. No activity was detected in the absence of Ca^{2+} (●). The inset shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of the myosin preparation (10 μg; lane A) and an autoradiogram (lane B) following phosphorylation of myosin in the presence of Ca^{2+}/calmodulin, myosin light chain kinase and Mg[γ-^{32}P]ATP^{2-}. HC = myosin heavy chain; LC_{20} and LC_{17} = the 20 kD and 17 kD light chains respectively.

centrations of felodipine and its analogues on the actin-activated myosin Mg^{2+}-ATPase. Felodipine and the *p*-chloro analogue inhibited this ATPase activity with IC_{50} = 25.1 μM. No significant inhibition by the oxidized and *t*-butyl analogues was observed up to 55.6 μM. Inhibition of actin-activated myosin Mg^{2+}-ATPase activity always correlated with inhibition of superprecipitation ($A_{660\,nm}$) (data not shown). The ~2-fold higher IC_{50} for inhibition of ATPase activity compared with inhibition of MLCK activity (Figs. 6 and 7) may be due to a small amount of drug binding to actin and/or tropomyosin which would lower the effective drug concentration in the ATPase experiments.

Myosin filament assembly. The reversible assembly of myosin into filaments is depicted in Fig. 9. The addition of ATP to non-phosphorylated smooth muscle myosin caused pre-existing myosin filaments to disassemble (shown by a reduction in $A_{340\,nm}$). Subsequent addition of Ca^{2+} and calmodulin activated the myosin light chain kinase (added to the system at the outset) and led to myosin phosphorylation and filament assembly. This was reversed by the action of myosin light chain phosphatase, which was facilitated by the addition of EGTA to inactivate the kinase.

Having verified the validity of this system, it was then possible to investigate the effects of felodipine and its analogues on the assembly of myosin filaments (Fig. 10). These experiments were carried out at a low concentration (5 nM) of calmodulin, which was sufficient to induce myosin filament assembly at a reasonable rate (Fig. 10a). Addition of felodipine or the *p*-chloro analogue (22.2 μM) with this calmodulin completely blocked the assembly of myosin into filaments. Addition of more calmodulin (1 μM) led to rapid filament assembly (Fig. 10a). On the other hand, neither the oxidized nor the *t*-butyl analogue prevented filament assembly induced by 5 nM calmodulin (Fig. 10b).

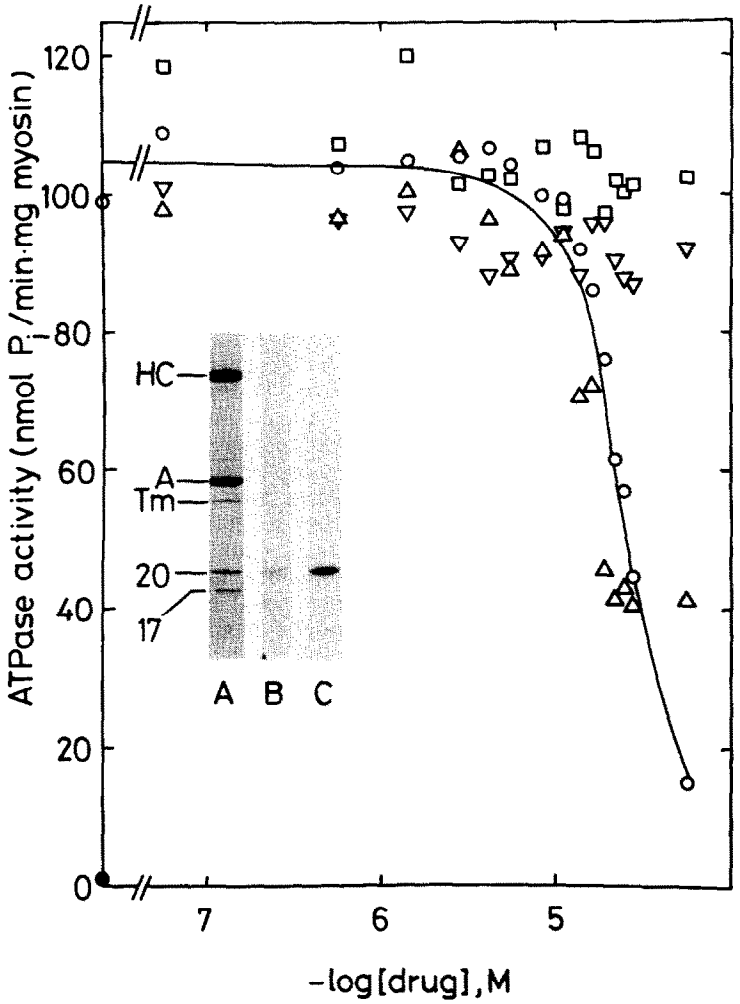

Fig. 8. Effects of felodipine and its analogues on the actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin. Actin-activated myosin Mg^{2+}-ATPase activity was measured, as described in Materials and Methods, in a reconstituted system composed of purified actin, myosin, tropomyosin, calmodulin and myosin light chain kinase, in the presence of various concentrations of felodipine (○) and its analogues: *p*-chloro (△), oxidized (▽) and *t*-butyl (□). The low ATPase activity observed in the absence of Ca^{2+} is indicated (●). The inset shows a Coomassie Blue-stained SDS/polyacrylamide gel electrophoretogram of the reconstituted system (A) and autoradiograms corresponding to the highest doses of felodipine (B) and the *t*-butyl analogue (C). HC = myosin heavy chain; A = actin; Tm = β subunit of tropomyosin (the γ subunit comigrates with actin in this electrophoretic system); 20 and 17 = the 20 kD and 17 kD light chains of myosin respectively.

DISCUSSION

Ca^{2+} channel blocking drugs such as felodipine and related dihydropyridines are effective vasodilating agents. Their abilities to inhibit smooth muscle contraction are thought to be due largely to their effects on the kinetics of Ca^{2+} influx through Ca^{2+} channels [3], but several observations have suggested an additional, intracellular mechanism of action of these compounds. For example, Ca^{2+} antagonists have been shown to induce relaxation in several smooth muscle preparations at doses lower than those required to inhibit Ca^{2+} influx or action potentials [4, 36]. Felodipine inhibits contractile activity to a greater extent than electrical membrane discharge, suggesting inhibition of action potential discharge but also a later (intracellular) step in the control of contractions in the rat portal vein [1]. Furthermore, Ca^{2+} antagonists could inhibit contractions induced in the absence of extracellular Ca^{2+} [37], and felodipine, in concentrations that prevented contraction, does not influence cellular uptake of $^{45}Ca^{2+}$ [4] or $^{45}Ca^{2+}$ binding and accumulation in isolated vascular sarcolemmal vesicles [1]. Finally, whereas the membrane-permeable Ca^{2+} antagonist methoxyverapamil is effective whether applied extra- or intracellularly, its membrane-impermeable quaternary amine derivative is effective only following intracellular injection [38]. Similarly, the charged pyridinium analogue of nifedipine is quite ineffective in inhibiting the response of guinea pig ileal longitudinal smooth muscle to a supramaximal muscarinic stimulation [39]. It is possible, however, that dihydropyridine Ca^{2+} channel blockers may exert

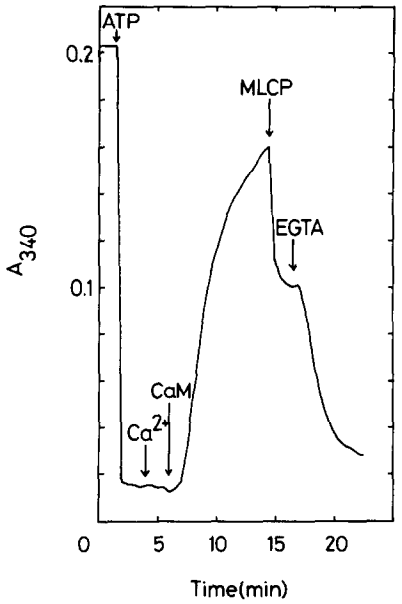

Fig. 9. Myosin filament assembly–disassembly. The reversible assembly of smooth muscle myosin into filaments was verified as described in Materials and Methods. Filament assembly–disassembly was monitored by recording the A_{340nm} of the solution. Pre-formed filaments of non-phosphorylated myosin were induced to disassemble by addition of ATP, as shown by the decrease in A_{340nm}. Subsequent addition of Ca^{2+} and calmodulin activated the myosin light chain kinase (present with the myosin from the outset), leading to myosin phosphorylation and filament reassembly. Subsequent addition of myosin light chain phosphatase and EGTA (to inactivate the kinase) resulted in myosin dephosphorylation and filament disassembly. The reversible phosphorylation of myosin was verified by urea/polyacrylamide gel electrophoresis.

their effects by binding to an intracellular site on the channel.

The dihydropyridines are markedly hydrophobic compounds and therefore are able to penetrate the sarcolemma easily and accumulate within the cell. Studies of [^{14}C]felodipine uptake indicated a tissue: medium ratio of 200:1 to 500:1 [1]. The plasma concentration of felodipine is 5–30 nM during antihypertensive therapy in patients [40] as well as in spontaneously hypertensive rats [41]. Intracellular concentrations of felodipine up to ~15 μM, therefore, can occur. Felodipine was found to bind to a protein of M_r 15–20 kD present in a crude actomyosin preparation derived from porcine aorta, and Ca^{2+}-dependent binding of felodipine to isolated calmodulin was demonstrated [4] with two binding sites per mol felodipine and $K_d = 8.5$ to 22 μM [5, 42]. Calmodulin plays a central role in the regulation of smooth muscle contraction through its regulation of myosin light chain kinase activity [43, 44]. Stimulation of the cell leads to an elevation of sarcoplasmic [Ca^{2+}], whereupon Ca^{2+} binds to calmodulin inducing a conformational change in the Ca^{2+}-binding protein. In its altered conformation, Ca_4^{2+}·calmodulin can interact with and activate the enzyme myosin light chain kinase which catalyzes the phosphorylation of serine-19 on each of the two 20 kD light chains of smooth muscle myosin. This phosphorylation leads to rapid cross-bridge cycling and the development of tension. Biochemically, myosin phosphorylation results in actin-activation of the myosin Mg^{2+}-ATPase and superprecipitation (the increase in turbidity observed as actin and myosin filaments interact). Blockage of calmodulin-myosin light chain kinase interaction by calmodulin antagonists could then prevent target enzyme activation and smooth muscle contraction. Such a possible additional mechanism of action of felodipine was originally suggested by Boström *et al.* [4].

Calmodulin is known to regulate the activities of several enzymes in addition to myosin light chain kinase. Two enzymes that have been implicated in the regulation of smooth muscle contraction are cAMP phosphodiesterase [45] and caldesmon kinase [21, 46]. Inactivation of both of these enzymes, like myosin light chain kinase, would be predicted to favour the relaxed state of the muscle.

In this study, we have examined the effects of felodipine and three analogues (*p*-chloro, oxidized and *t*-butyl) on the activities of these three calmodulin-dependent enzymes: cAMP phosphodiesterase, caldesmon kinase and myosin light chain kinase. These four compounds differ in pharmacological potency: pIC_{50} ($-\log IC_{50}$) values for inhibition of contraction of rat portal vein were 7.4 (felodipine), 5.2 (*p*-chloro analogue), 4.6 (oxidized felodipine) and <4.5 (*t*-butyl analogue) (B. Ljung, personal communication, cited with permission). The two weakest analogues (oxidized and *t*-butyl derivatives) had little, if any, effect on the activities of the three calmodulin-dependent enzymes, nor did they affect actin-activated myosin Mg^{2+}-ATPase activity, superprecipitation, or myosin filament assembly. Felodipine, however, inhibited cAMP phosphodiesterase, caldesmon kinase, myosin light chain kinase and actin-activated myosin Mg^{2+}-ATPase, as well as myosin filament assembly induced by a low concentration of calmodulin. The IC_{50} values were similar to the concentrations of felodipine which can occur intracellularly *in vivo* (see above). The *in vitro* inhibitory effects of felodipine are therefore specific and qualitatively resemble the pharmacological effects on the contractile properties of the rat portal vein. Structural alterations such as oxidation of the dihydropyridine ring or replacement of the methyl and ethyl ester side chains with *t*-butyl groups result in a substantial loss of pharmacological potency and ability to inhibit calmodulin-dependent enzymes and processes. However, movement of the *ortho* chlorine atom of felodipine to the *para* position results in a significant loss of pharmacological potency, but no loss of capacity to inhibit calmodulin-dependent enzymes and processes. The reason for this discrepancy is unknown, although it may reflect the fact that the primary pharmacological site of action of felodipine is the voltage-operated Ca^{2+} channel, whereas calmodulin and calmodulin-regulated enzymes represent a secondary, and quantitatively less important, site of action.

A correlation has been observed between the affinities of felodipine and a variety of structural analogues for calmodulin and the hydrophobicities of the compounds (J. D. Johnson, personal com-

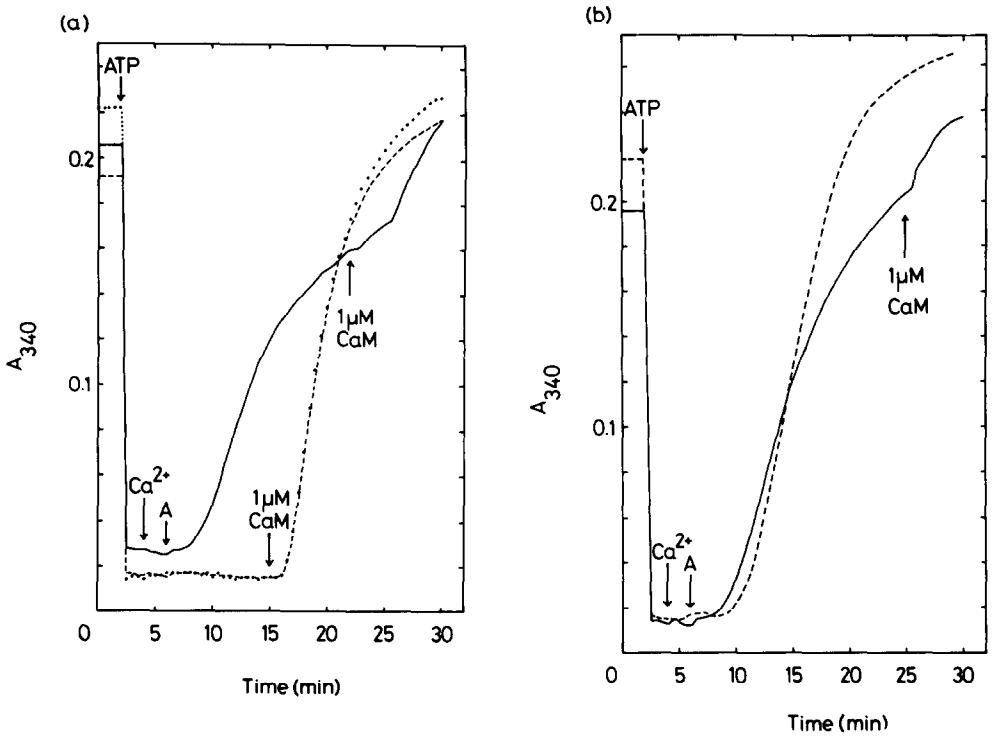

Fig. 10. Effects of felodipine and its analogues on myosin filament assembly. Myosin filaments were disassembled, under conditions described in Materials and Methods, by the addition of ATP. (a) Ca^{2+} was added as indicated, followed (at "A") by 5 nM calmodulin alone (———) or in combination with 22.2 μM felodipine (– – –) or 22.2 μM *p*-chloro analogue (· · · ·). (b) Ca^{2+} was added as indicated, followed (at "A") by 5 nM calmodulin in combination with 22.2 μM oxidized felodipine (———) or 22.2 μM *t*-butyl analogue (– – –). Excess calmodulin (1 μM) was added as indicated.

munication, cited with permission). Thus, the *t*-butyl analogue has a much higher affinity ($K_d = 0.3$ μM) for calmodulin than does felodipine ($K_d = 9$ μM). It is clear that no such correlation exists between hydrophobicity and capacity to inhibit calmodulin-stimulated enzymes and processes, or contraction of rat portal vein smooth muscle. Studies of tryptic digestion of calmodulin in the presence of bound drugs suggest that felodipine and the *t*-butyl analogue bind to different sites on calmodulin, which could explain why the *t*-butyl analogue binds tightly to calmodulin but has no effect on its ability to activate target enzymes (H. Vogel, personal communication, cited with permission).

The results described herein support a possible mechanism of action of felodipine which involves its Ca^{2+}-dependent interaction with calmodulin and consequent inhibition of calmodulin-dependent enzymes such as cAMP phosphodiesterase, caldesmon kinase, and myosin light chain kinase. Such inhibitory effects would favour the relaxed state of the smooth muscle and therefore vasodilation.

In conclusion, current understanding of the mechanism of action of felodipine indicates a primary action of the dihydropyridine on voltage-operated Ca^{2+} channels with additional, secondary sites of action within the vascular smooth muscle cell. Calmodulin and calmodulin-regulated enzymatic systems may represent one such secondary site of action of felodipine and related dihydropyridines.

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