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Direct inhibitory effects of some 'calcium-antagonists' and trifluoperazine on the contractile proteins in smooth muscle

M. Spedding

Centre de Recherche Merrell International, 16, rue d'Ankara, 67084 Strasbourg-Cedex, France

1 Taenia preparations from the guinea-pig caecum were treated with Triton X-100 and glycerol to disrupt the plasma membrane. Disruption of the sarcolemma was confirmed by electronmicroscopy. The preparations contracted in response to low concentration of Ca^{2+} (10–40 μM) and the contractions were dependent upon exogenous adenosine triphosphate (ATP).

2 Nifedipine (100 μM), verapamil (100 μM) and diltiazem (100 μM) did not inhibit Ca^{2+}-induced activation of the contractile proteins.

3 In contrast, fendiline (100 μM), cinnarizine (100 μM), flunarizine (100 μM), pimozide (100 μM) and trifluoperazine (100 μM) significantly inhibited Ca^{2+}-induced contractions. The effects of cinnarizine (100 μM) were reversible.

4 These findings disclose further differences between calcium-antagonists and suggest that certain of these agents have an intracellular site of action.

Introduction

Drugs classed as calcium-antagonists (Fleckenstein, Tritthart, Fleckenstein, Herbst & Grün, 1969; Godfraind & Kaba, 1969; Fleckenstein, 1977; Quintana, 1978; Godfraind, 1981) are thought to inhibit contractility in smooth muscle by selective inhibition of voltage-dependent Ca^{2+} channels and the term calcium-entry blocker has been proposed as a more precise description of these drugs (Vanhoutte, 1981). However, there is disagreement as to whether all these agents inhibit $^{45}Ca^{2+}$ influx in smooth muscle (Weston, 1978; Thorens & Haeusler, 1979; Church & Zsotér, 1980). Moreover, different binding sites for calcium-antagonists have been demonstrated (Ehlert, Roeske, Itoga & Yamamura, 1982; Murphy & Snyder, 1982). I have proposed recently a classification of calcium-antagonists into three distinct subgroups on the basis of structural differences between the drugs and functional differences both *in vitro* (Spedding, 1982a) and *in vivo* (Spedding, 1981; 1982b). One of these subgroups consists of lipophilic, weakly basic drugs often associated with a benzhydryl moiety (Figure 1). These drugs are readily distinguished from other calcium-antagonists in K^+-depolarized smooth muscle in that the inhibitory effects are time- and Ca^{2+}-dependent and are not readily reversible (Spedding, 1982a). These findings could indicate an intracellular site of action.

Trifluoperazine is another lipophilic, weakly basic drug which has inhibitory effects on Ca^{2+}-induced contractions in K^+-depolarized smooth muscle (Spedding, 1982a). Trifluoperazine directly inhibits activation of the contractile proteins in skinned smooth muscle preparations (Cassidy, Hoar & Kerrick, 1980; Mrwa, Peterson & Rüegg, 1980; Sparrow, Mrwa, Hofmann & Rüegg, 1981) by binding to calmodulin (Sparrow *et al.*, 1981; see also Levin & Weiss, 1979) and preventing activation of the calmodulin-dependent myosin light chain kinase (Kerrick, Hoar & Cassidy, 1980). As there are structural similarities between the lipophilic calcium-antagonists and trifluoperazine (Figure 1) and as a second calcium-antagonist, pimozide, has also been shown to bind to calmodulin (Levin & Weiss, 1979) I have tested a range of calcium-antagonists for direct inhibitory effects on the contractile proteins of smooth muscle, using taenia preparations from the guinea-pig caecum in which the sarcolemma had been chemically disrupted (Gordon, 1978; Sparrow *et al.*, 1981). Some of these results have been communicated to the British Pharmacological Society (Spedding, 1982c).

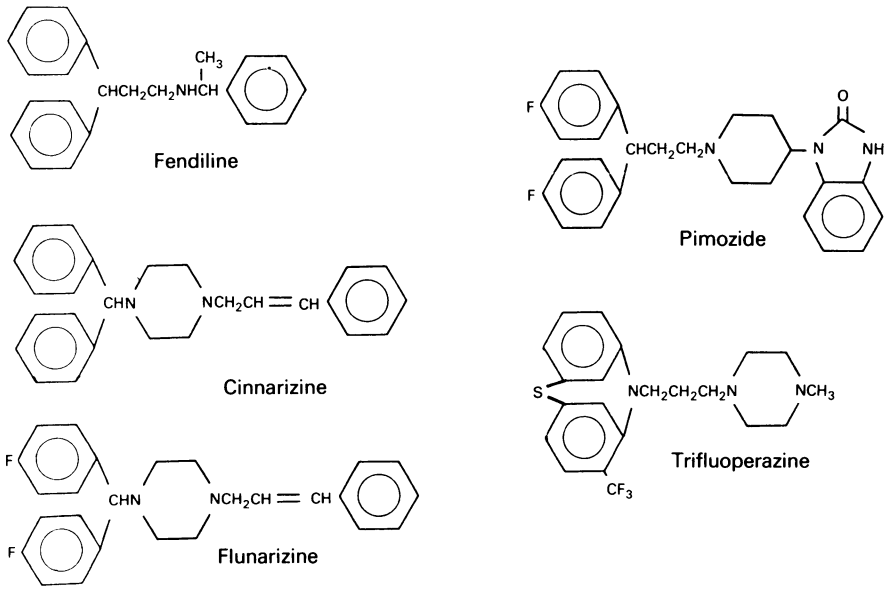

Figure 1 Comparison of the structures of lipophilic calcium-antagonists with trifluoperazine.

Methods

Thin (approx. 0.5 mm diameter) strips of longitudinal smooth muscle were cut from the taenia of the caecum from male guinea-pigs (200–350 g). In some experiments unskinned taenia preparations were set up in isolated organ baths in Tyrode solution (composition, mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.1, $CaCl_2$ 1.8, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, atropine 0.0017; pH 7.3) maintained at 35°C and gassed with 95% O_2:5% CO_2. Resting tension was set at 0.5 g. Repeated isometric contractions were obtained by addition of K^+ (60 mM for 5 min at 30 min intervals) and recorded using Grass FT03 transducers and displayed on a Grass 79D polygraph.

Skinned taenia preparations

The taenia were chemically skinned of their cell membranes by the method of Sparrow *et al.* (1981). Preparations were initially dissected in Tyrode solution and were then incubated for 4 h at 4°C in a buffer containing Triton X-100 (composition, mM: imidazole 20, ethyleneglycol *bis*-amino-ethylether N,N′-tetraacetic acid (EGTA) 5, KCl 50, sucrose 150, dithiothreitol 0.5, Triton X-100 1%, pH 7.4). After a 15 min rinse in Triton-free buffer the strips were stored for 5–10 days at −20°C in glycerol/buffer (composition, mM: imidazole 20, EGTA 4, $MgCl_2$ 10, adenosine 5′-triphosphate (ATP) 7.5, sodium azide 1, dithiothreitol 0.5, glycerol 50%, pH 6.7). The preparations were then cut into 10 mm lengths and set up in isolated organ baths containing a Ca^{2+}-free buffer (composition, mM: imidazole 10, EGTA 4, $MgCl_2$ 10, ATP 7.5, sodium azide 1, K_2PO_4 6, pH 6.7) at 20–22°C. The preparations were gassed with O_2 to facilitate mixing. Resting tension was set at 0.5 g and isometric contractions were recorded as above. Contractions were induced at 30–40 min intervals by adding $CaCl_2$ to give a Ca^{2+} concentration of 10–40 μM (Sparrow *et al.*, 1981) using the equations described by Portzehl, Caldwell & Rüegg (1964) and the apparent binding constant for Ca^{2+}/EGTA of 1.2×10^{-6} M.

Drugs

The following drugs were used: adenosine 5′-triphosphate, disodium salt (Sigma); atropine sulphate (Sigma); calmodulin (Sigma); cinnarizine tartrate (Janssen Pharmaceutica); diltiazem hydrochloride (Synthelabo); 2,4-dinitrophenol (Sigma) 1,4-dithiothreitol (Merck); EGTA (Sigma); fendiline hydrochloride (Dr Thiemann GmbH); flunarizine hydrochloride (Janssen Pharmaceutica); imidazole (Sigma); inosine 5′-triphosphate (Boehringer Ingelheim); nifedipine (Bayer AG); pimozide tartrate (Janssen Pharmaceutica); trifluoperazine (Terfluzine: Theraplix); Triton X-100 (BDH); (±)-verapamil hydrochloride (Knoll AG).

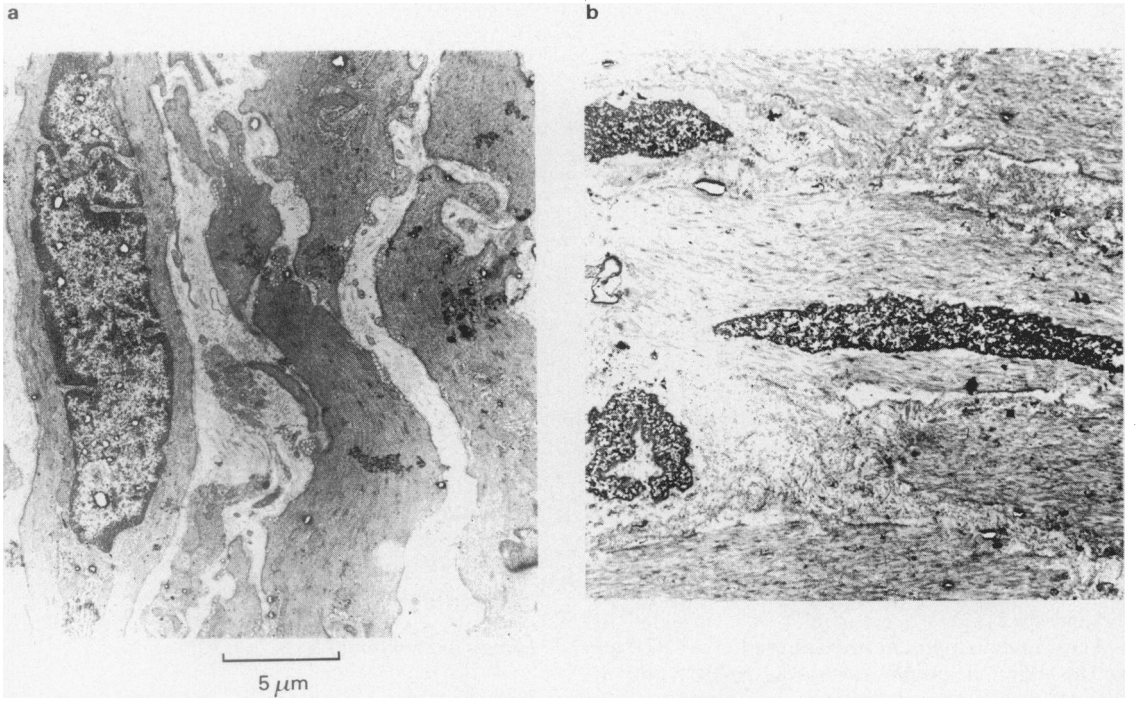

Figure 2 Comparison of the morphology of taenia preparations from the guinea-pig caecum maintained in Tyrode solution (a), with preparations which had been skinned by the method of Sparrow *et al.* (1981) (b). The preparations were fixed in 25% glutaraldehyde for 60 min at 4°C and subsequently in 2% osmic acid for 60 min at 4°C, prior to embedding in resin for electronmicroscopy.

Results

Histological changes in skinned muscle

Figure 2 shows a comparison of the morphology of a taenia preparation which had been prepared for electronmicroscopy following a 60 min incubation in Tyrode solution with a preparation which had been pretreated with Triton and stored in 50% glycerol at 20°C for seven days. Whereas the control preparations had intact cell membranes with abundant caveolae, the Triton-treated preparations did not have clearly defined cell boundaries, indicating almost complete disruption of the sarcolemma. The disruption was uniform in all the sections studied and was not confined to the outermost regions of the strips.

Contractile properties of skinned muscle

The taenia preparations which had been pretreated with Triton contracted in response to low concentrations of Ca^{2+} (10–40 μM, Figure 3). The concentration-response curve was steep. A maximum response was always obtained with 40 μM, but the threshold varied between 10–20 μM. Therefore the preparations were routinely contracted with Ca^{2+} 20 μM, at 30–40 min intervals (Figure 4). Under these conditions there was a slow decline (~20%) in contractility over 3 h. The preparations were dependent upon exogenous ATP as an energy source as omission of ATP from the incubation medium resulted in a slowly developing rigor contraction which was independent of Ca^{2+}. Unlike skinned cardiac muscle preparations (Toyo-Oka, 1981), it was possi-

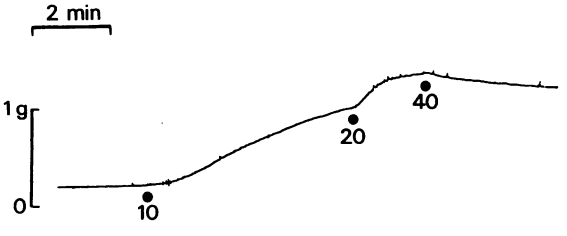

Figure 3 Concentration-response curve of a skinned taenia preparation from the guinea-pig caecum to Ca^{2+} (μM).

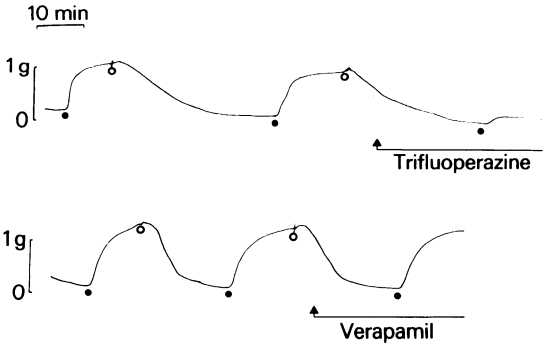

Figure 4 Effects of trifluoperazine (100 μM) and verapamil (100 μM) on the contractile responses of skinned taenia preparations from the guinea-pig caecum to Ca^{2+} (● 20 μM; ○ washout).

ble to substitute inosine 5′-triphosphate (ITP) for ATP without development of a contraction, and without affecting Ca^{2+} sensitivity. The Ca^{2+}-induced contractions were not affected by 2,4-dinitrophenol (55 μM, $n = 3$).

Tension development in response to Ca^{2+} (20 μM) in the skinned preparations was significantly increased when the preparations were pretreated for 3 h with calmodulin, 0.2 μM (control strips 0.95 ± 0.12 g, paired pretreated strips 1.67 ± 0.19 g, $n = 4$, $P < 0.05$, paired t test). However, no effect was seen following short (10–30 min) incubation periods with calmodulin (0.2–1 μM).

Effects of calcium-antagonists

The protocol for assessment of inhibitory effects of the antagonists is depicted in Figure 4. Under these conditions, 15 min incubation with high concentrations (100 μM) of trifluoperazine inhibited Ca^{2+}-induced contractions whereas verapamil was ineffective. The effects of several calcium antagonists on Ca^{2+}-induced activation of the contractile proteins are presented in Figure 5. Whereas nifedipine, verapamil and diltiazem did not significantly inhibit Ca^{2+}-induced contractions, the lipophilic drugs (fendiline 100 μM, cinnarizine 100 μM, flunarizine 100 μM, trifluoperazine 100 μM, pimozide 100 μM) did have inhibitory effects. The effects of cinnarizine (100 μM) were significantly ($P < 0.05$) greater than those of trifluoperazine (100 μM). The inhibitory effects of cinnarizine (100 μM) were optimal within the 20 min incubation period and did not increase following prolonged incubation if the slow decline in contractility in the control preparations is taken into account (Figure 6). Furthermore the inhibition was reversed following washout of the drug (Figure 6).

Effects in unskinned preparations

The inhibitory effects of the antagonists were also assessed against K^+ (60 mM)-induced contractions of unskinned taenia preparations maintained in Tyrode solution (Figure 7). Nifedipine (10 μM), verapamil (100 μM), diltiazem (100 μM) and pimozide (100 μM) abolished K^+-induced contractions after a 20 min

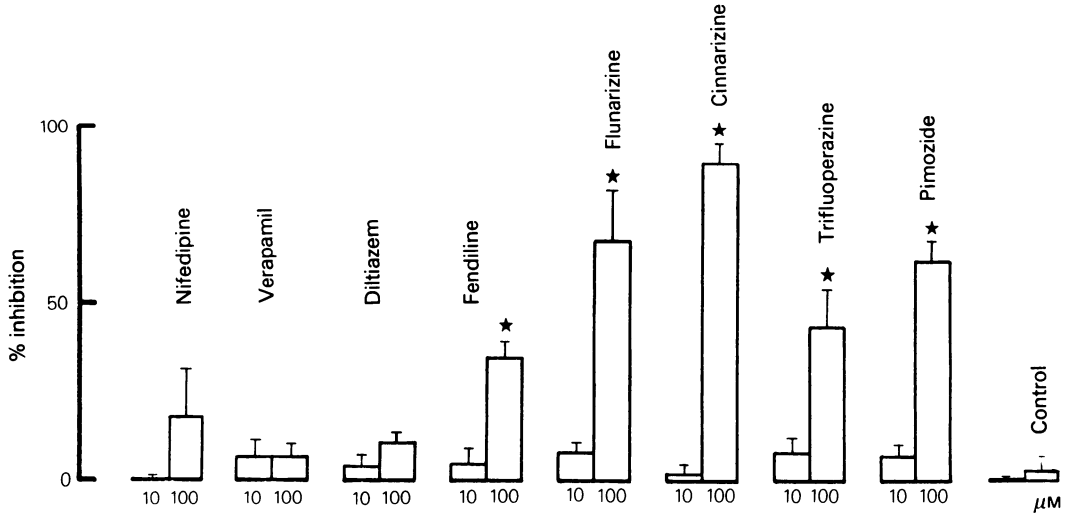

Figure 5 Inhibitory effects of 20 min incubation with calcium-antagonists (10 or 100 μM) on the contractile responses of skinned taenia preparations from the guinea-pig caecum to Ca^{2+} (20 μM). Vertical lines represent s.e.mean, n is 5–8. *Significant inhibition compared with control ($P < 0.01$ for fendiline, otherwise $P < 0.001$).

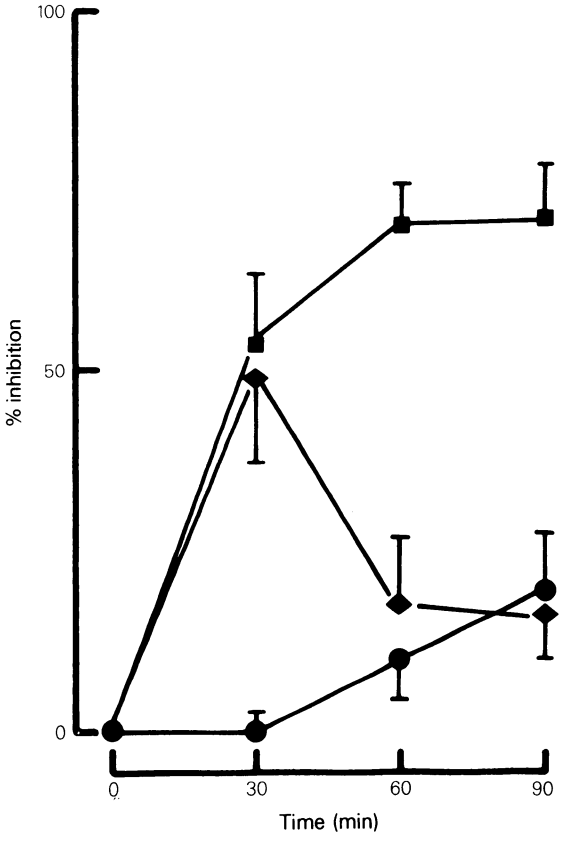

Figure 6 Inhibitory effects of cinnarizine (100 μM) on the responses of skinned taenia preparations from the guinea-pig caecum to Ca^{2+} (20 μM). Cinnarizine was left in contact with the tissue for three contractions (■) or washed out, with the Ca^{2+}, after the first contraction (▲), i.e. after 20 min incubation only. The effects of cinnarizine did not significantly increase following incubation after the first 20 min if the decline in contractility seen in the control preparations (●) is subtracted. Vertical bars represent s.e.mean ($n = 4$).

incubation period and fendiline (100 μM) and trifluoperazine (100 μM) significantly ($P < 0.05$) inhibited K^+-induced contractions. In marked contrast, flunarizine (100 μM) and cinnarizine (100 μM) were without effect.

Discussion

Properties of skinned muscle

There can be little doubt that contraction of the skinned taenia preparations in response to addition of Ca^{2+} results from direct activation of the contractile proteins. Histological examination of the preparations showed that the sarcolemma was disrupted and the cells were permeable to large molecules because pretreatment with calmodulin augmented the contractions, as has been shown previously by Sparrow *et al.* (1981). Furthermore, the preparations were sensitive to very low concentrations of Ca^{2+} and were dependent upon exogenous ATP.

However, the strips were not as sensitive to calmodulin as those described by Sparrow *et al.* (1981). Thus, these authors found addition of calmodulin to cause an immediate increase in sensitivity to Ca^{2+} whereas the preparations used in the present study had to be preincubated with calmodulin for 3 h. This discrepancy may be due to different sources of calmodulin, or to the greater thickness of the strips used in this study. As a result, calmodulin could not be used to reverse the effects of the antagonists. It is likely that the strips were deficient in soluble proteins, including calmodulin, after the skinning procedure because similar procedures have been used to extract soluble proteins from vascular smooth muscle (e.g. myosin light chain kinase; Vallet, Molla & Demaille, 1981).

Calcium-antagonists in skinned muscle

Nifedipine, diltiazem and verapamil did not affect contractility in skinned taenia preparations. Thus these compounds do not interact with the contractile proteins (Nayler & Poole-Wilson, 1981) and must therefore act at, or close to, the sarcolemma, although the site of action for verapamil and diltiazem is different from the site occupied by dihydropyridine calcium-antagonists (Ehlert *et al.*, 1982, Murphy & Snyder, 1982; see Spedding, 1982b). In contrast, the other drugs inhibited Ca^{2+}-induced activation of the contractile proteins. Cinnarizine caused greater inhibition than did trifluoperazine. The low sensitivity

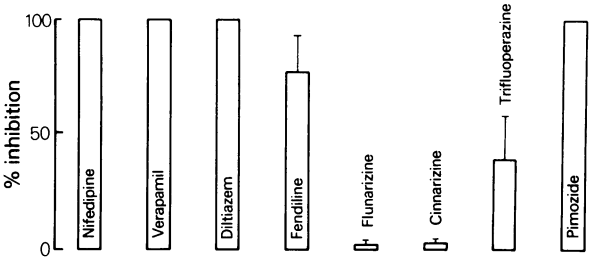

Figure 7 Inhibitory effects of 20 min incubation with calcium-antagonists (nifedipine 10 μM; otherwise 100 μM) on the contractile responses to K^+ (60 mM) in unskinned taenia preparations from the guinea-pig caecum maintained in Tyrode solution. Note that cinnarizine and flunarizine were devoid of inhibitory effects in these experiments. Vertical bars represent s.e.mean ($n = 4-6$).

of the strips to calmodulin precluded definition of the precise site of the inhibitory effects. However, it seems likely that the effects of the lipophilic drugs are mediated by binding to calmodulin as the interaction of trifluoperazine with calmodulin has been shown to be a lipophilic interaction with a hydrophobic part of what is one of the most acidic proteins in the cytoplasm (Landry, Amellal & Ruckstuhl, 1981; Roufogalis, 1981).

The inhibitory effects of these drugs were only seen at high concentrations (100 μM). Other workers who have defined the role of trifluoperazine in skinned smooth muscle have had to use similarly high concentrations to demonstrate inhibitory effects (Cassidy *et al.*, 1980; Kerrick *et al.*, 1980; Mrwa *et al.*, 1980, Sparrow *et al.*, 1981). Thus the important question arises as to whether the inhibitory effects of these drugs on Ca^{2+}-induced contractions in skinned smooth muscle bear any relationship to the inhibitory effects against Ca^{2+}-induced contractions in K^+-depolarized smooth muscle, which occur at much lower concentrations (0.1–10 μM, Spedding, 1982). Although the discrepancy between the concentrations required for inhibition of the two types of Ca^{2+}-induced contractions is considerable there are several reasons why the two effects may be interrelated.

First, Levin & Weiss (1979) have shown that pimozide and trifluoperazine bind to purified calmodulin in the low concentrations which produce inhibitory effects in K^+-depolarized smooth muscle (0.1 and 1 μM). Trifluoperazine is less effective when calmodulin is tightly bound to its binding proteins and it is likely that the calmodulin remaining in skinned muscle after the skinning procedure will be so sequestered and thus resistant to the effects of the drugs.

Second, the high potency of the lipophilic drugs in K^+-depolarized preparations is seen only when the antagonists are incubated in Ca^{2+}-free media (Spedding, 1982a); the drugs are less potent when incubated with unskinned preparations in the presence of Ca^{2+}. Figure 6 shows that the same high concentrations (100 μM for 20 min) of flunarizine and cinnarizine which inhibited contractility in skinned muscle were ineffective against K^+-induced contractions of unskinned taenia preparations maintained in Tyrode solution, although inhibitory effects were observed following longer incubation periods (120 min, unpublished observations). These remarkable discrepancies in potency may be accounted for if the cell membrane hinders access to the site of action of these drugs. Potency in unskinned preparations would therefore be a reflection both of the complex factors governing intracellular accumulation and of the affinity for the site of action. Such considerations would be consistent with the apparent irreversible nature of the antagonist effects of cinnarizine on Ca^{2+}-induced contractions in K^+-depolarized smooth muscle preparations (Spedding, 1982a) compared with the readily reversible effects on Ca^{2+}-induced contractions in skinned smooth muscle.

Finally, the contractile proteins may be only one of several sites of action for drugs such as trifluoperazine, or cinnarizine. Morel, Wibo & Godfraind (1981) have demonstrated a calmodulin-stimulated Ca^{2+}-pump in the plasma membrane of rat aorta and these workers have also shown selective inhibition by cinnarizine and flunarizine, but not verapamil, of a calcium-activated ATPase in microsomes from this tissue (Morel & Godfraind, 1978). Although it may seem surprising, inhibition of Ca^{2+} ATPases in smooth muscle and consequently increased cytosolic Ca^{2+} could lead to *secondary* inhibition of $^{45}Ca^{2+}$ entry if the voltage-dependent Ca^{2+} channels in smooth muscle are inhibited by cytosolic Ca^{2+}, as is the case in *Aplysia* neurones (Eckert & Ewald, 1982).

In conclusion, this paper demonstrates that some, but not all, calcium-antagonists inhibit activation of the contractile proteins, albeit in high concentrations. These findings re-emphasize the differences which exist between calcium-antagonists and are compatible with the classification of these drugs into three distinct subgroups which I have previously proposed (Spedding, 1982a, b). Thus, the lipophilic agents shown in Figure 1 constitute one of the subgroups and can be clearly differentiated from verapamil, diltiazem and the dihydropyridine calcium-antagonists in skinned smooth muscle.

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