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Changing surface charge with salicylate differentiates between subgroups of calcium-antagonists

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1 Sodium salicylate (5–10 mM) has been used to distinguish the effects of the three calcium-antagonist subgroups which had been previously differentiated in functional studies.

2 Sodium salicylate (10 mM) reduced the antagonistic effects of verapamil and diltiazem on Ca^{2+}-induced contractions of K^+ (40 mM)-depolarized taenia preparations from the guinea-pig caecum. In contrast, salicylate had no effect on the potency of nifedipine and increased the inhibitory effects of cinnarizine and flunarizine. Sodium salicylate (10 mM) had little effect on Ca^{2+}-induced contractions *per se*.

3 In preparations pretreated with calcium-antagonists and recontracted with high concentrations of Ca^{2+}, salicylate (5 mM) caused an additional contraction when the preparations had been pretreated with verapamil or diltiazem but had no effect in control or nifedipine-treated preparations. In contrast, salicylate relaxed Ca^{2+}-induced contractions in tissues which had been pretreated with cinnarizine, flunarizine, pimozide, bepridil, fendiline, perhexiline and with the calmodulin antagonist W-7.

4 The mechanism of action of salicylate was investigated. Inhibition of prostaglandin biosynthesis or of oxidative phosphorylation by salicylate was not responsible for these effects because indomethacin (28 μM) and 2,4-dinitrophenol (20 μM) did not differentiate between calcium antagonists. The effects of salicylate are ascribed to an increase in negative surface charge on the membrane because other agents changing surface charge (3,5-dichlorosalicylate, 0.3 mM; benzoate, 20 mM) have similar effects and their potency is dependent on their affinity for lipid membranes. Furthermore, salicylate increased the effectiveness of the cationic local anaesthetic, (+)-propranolol (100 μM), but did not change the effects of the neutral local anesthetic, benzocaine (1 mM).

5 It is argued that salicylate increases the effectiveness of cinnarizine by increasing accumulation of this drug in the cell membrane or at intracellular sites whereas the reduced effectiveness of verapamil and diltiazem is secondary to a change in the state of the Ca^{2+} channel.

Introduction

The chemically-disparate group of drugs which act as calcium-antagonists have been claimed to comprise several subgroups on the basis of binding studies (Ferry & Glossmann, 1982) and of structural and functional differences between the compounds (Fleckenstein, 1981; Spedding, 1981; 1982a,b; 1983, Table 1). The diphenylalkylamines are the most lipophilic of these agents and can be readily differentiated from verapamil, diltiazem and the dihydropyridines in K^+-depolarized smooth muscle because their inhibitory effects are reduced by the presence of Ca^{2+} during the incubation period (Spedding, 1982a).

A possible explanation for the protective effect of

Table 1 Literature classification of calcium antagonists

	Dihydropyridines[1]	*Verapamil*	*Diltiazem*	*Diphenylalkylamines*[2]
Fleckenstein (1981)	A	A	A	B
Spedding (1981, 1982a,b, 1983)	A	B	B	C
Ferry & Glossmann (1982)	I_A	II	III	I_B

[1] e.g. nifedipine, nicardipine, PY 108–068.
[2] e.g. cinnarizine, flunarizine, fendiline, pimozide, prenylamine.

Ca^{2+} is that the cation may shield negative surface charges on cell membranes. There are 15–20 mEq kg^{-1} of fixed negative surface charge in guinea-pig taenia preparations (Brading & Widdicombe, 1977) with different affinities for divalent or monovalent cations (Goodford & Wolowyk, 1972; Brading, 1981). Surface charges affect ion channel gating by locally contributing to the electrical field across the membrane (see Hille *et al.*, 1975). In addition a small number of charges at or in ion channels will control ion selectivity for the channel. Thus some surface charges will have specific effects on ion channels whereas the general surface charge of the membrane will have more widespread effects, such as influencing cation binding and the affinity of charged compounds for the membrane.

McLaughlin (1973) has shown that salicylate is adsorbed onto lipid bilayers giving an increased surface charge to the bilayer; the negative charge increased the passage of cationic drugs through the membrane. Effects of salicylate on surface charge have also been described using electrophysiological techniques in molluscan neurones (Levitan & Barker, 1972) and in sheep ventricular fibres (Cohen *et al.*, 1979), although in these studies surface charge was measured indirectly from changes in K^+ permeability. This paper describes the effects of increasing membranal negative surface charge with salicylate ions on calcium-antagonist function. In addition the effects of salicylate on the ability of high concentration of (+)-propranolol and benzocaine to inhibit Ca^{2+}-induced contractions (Spedding & Berg, 1984, unpublished data) has been investigated; the effects of benzocaine should be independent of surface charge as benzocaine is a neutral local anaesthetic.

Methods

Strips of taenia (1–2 mm diameter, 20–25 mm relaxed length) from the caecum of male guinea-pigs (200–300 g) were set up in 10 ml isolated organ baths containing K^+ depolarizing Tyrode solution (mM: NaCl 97, KCl 40, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, pH 7.1), which was gassed with 95% O_2: 5% CO_2 and maintained at 35°C. Contractile responses were measured isotonically under 1 g load and responses were recorded using Harvard or Bioscience isotonic transducers connected to Rikadenki potentiometric recorders. Cumulative concentration-response curves to Ca^{2+} were constructed by increasing the bath concentration in logarithmic increments (Van Rossum, 1963). The curves were obtained after a 20 min washout period in Ca^{2+}-free solution; this procedure allows reproducible sensitivity to Ca^{2+} for several hours (Spedding, 1982a). The 100% response was taken as the maximum contraction (usually 3 mM Ca^{2+}) during the second concentration-response curve, the first curve being discarded from analysis. Dose-ratios were calculated as the ratio of the concentrations of Ca^{2+} which produced a 50% maximal response (EC_{50}) in the presence and absence of the antagonist.

In some experiments preparations were pretreated with calcium-antagonists for 20 min before the addition of Ca^{2+} (3 mM) which contracted the tissues to 35–65% of the maximal response. When the contraction had stabilized, salicylate was added and the % change in this response calculated, taking the steady state response as 100%.

Values in the text refer to means ± s.e.mean. Student's *t* test was used for comparisons. The mean

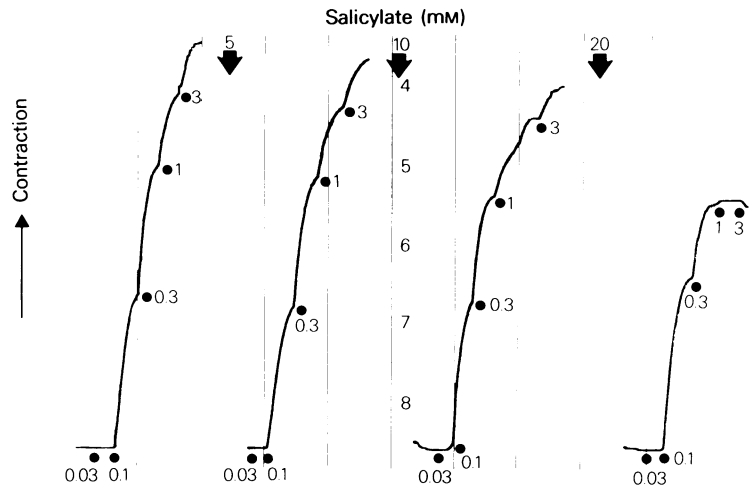

Figure 1 Inhibitory effects of sodium salicylate (5, 10 and 20 mM) on cumulative concentration-response curves to $CaCl_2$ (●, mM) in a taenia preparation from the guinea-pig caecum maintained in K^+ (40 mM)-Tyrode solution.

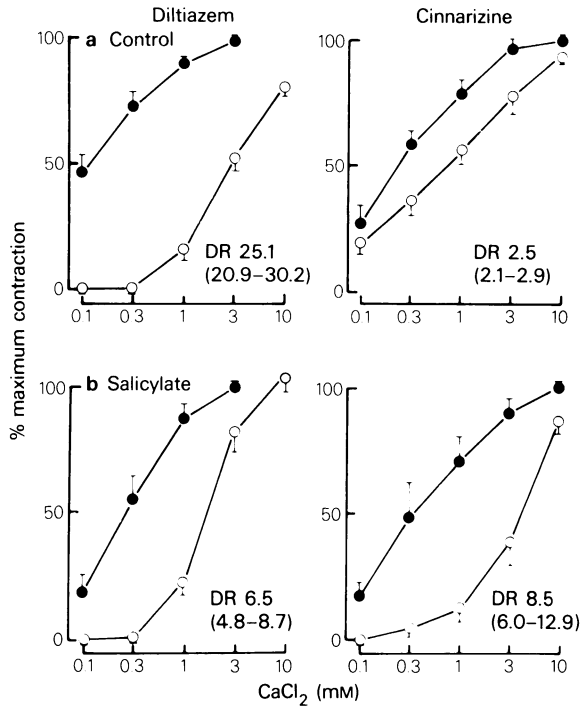

Figure 2 Antagonistic effects of diltiazem (1 μM for 20 min, left panels) and cinnarizine (1 μM for 20 min, right panels) on cumulative concentration-response curves to $CaCl_2$ (● control; ○ with antagonist) in K^+-depolarized taenia preparations. The upper panels were control preparations and the lower panels were paired preparations in the presence of sodium salicylate (10 mM). Vertical bars represent s.e.mean, $n = 5-7$.

dose-ratios were calculated as logarithms and these values were tested for statistical significance; the antilogarithms of these values are shown in the text, with the range of the s.e.mean.

Drugs

The following drugs were used: sodium salicylate, sodium benzoate (E. Merck), sodium 3,5-dichlorosalicylate (ICN Pharmaceuticals), cinnarizine tartrate, flunarizine hydrochloride, pimozide tartrate (Janssen Pharmaceutica); diltiazem hydrochloride, N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) (Synthelabo); bepridil (Organon); 2,4-dinitrophenol (Sigma); fendline hydrochloride (Dr Thiemann GmbH); indomethacin (MSD); nifedipine (Bayer AG); (±)-verapamil hydrochloride (Knoll). Indomethacin was dissolved in a solution of 1% sodium carbonate and nifedipine (1 mM) was dissolved in ethanol. All the drugs were diluted in distilled water before use.

Results

Salicylate pretreatment

Preincubation for 20 min with sodium salicylate (5–20 mM) did not markedly change the sensitivity of K^+ (40 mM)-depolarized taenia preparations to Ca^{2+}, although the maximum response of cumulative concentration-response curves was reduced, the effect being most prominent at 20 mM (Figure 1). In contrast, the calcium antagonists diltiazem (1 μM) and cinnarizine (1 μM) caused parallel displacement to the right of the cumulative concentration-response curves to Ca^{2+} (Figure 2, upper panels). However, if the antagonist were incubated in the presence of salicylate (10 mM for 20 min) the inhibitory effects of diltiazem were reduced, whereas the effects of cinnarizine were increased (Figure 2, lower panels).

The inhibitory effects of these and other calcium-antagonists, expressed as Ca^{2+} dose-ratios, in the absence and presence of sodium salicylate (10 mM for 20 min) are shown in Table 2. Salicylate reduced the

Table 2 Effectiveness of calcium-antagonists in the absence or presence of sodium salicylate (10 mM)

	Ca^{2+} dose-ratio (range s.e.mean)		
Antagonist	*Control*	*Salicylate*	n
Diltiazem, 1 μM	25.1 (20.9–30.2)	6.5 (4.8–8.7)*	5
Verapamil 0.2 μM	20.0 (14.4–27.5)	5.5 (3.5–6.9)*	5
Nifedipine, 3 nM	2.5 (2.3–2.6)	2.8 (2.3–3.5)	5
Nifedipine, 10 nM	64.7 (49.9–82.4)	47.0 (39.8–74.9)	8
Cinnarizine, 1 μM	2.5 (2.1–2.9)	8.5 (6.0–12.9)*	7
Flunarizine, 1 μM	2.8 (2.3–3.4)	10.2 (6.1–17.4)*	5

The calcium-antagonists were incubated with taenia preparations for 20 min in Ca^{2+}-free, K^+-depolarizing Tyrode solution before cumulative addition of Ca^{2+} (0.1–10 mM). The antagonistic effects are expressed as Ca^{2+} dose-ratios (range s.e.mean). Preparations in which salicylate (10 mM for 20 min) was added with the antagonist were run in parallel, comparing tissues from the same animal, $*P<0.05$.

inhibitory effects of verapamil and diltiazem and increased the inhibitory effects of flunarizine and cinnarizine. However, the inhibitory effects of nifedipine were unchanged by salicylate despite the use of concentrations of nifedipine causing small (3 nM) or substantial (10 nM) antagonism of Ca^{2+}-induced contractions.

Salicylate addition to established contractions

The effects of salicylate were also investigated in tissues previously incubated with calcium-antagonists. Salicylate (5 mM) added to established Ca^{2+}-induced contractions in control preparations did not cause a direct relaxation (Figure 3), although 10 mM had slight relaxant effects (20–40%). Calcium-antagonists were therefore preincubated with taenia preparations for 20 min in Ca^{2+}-free K^+-Tyrode solution and their antagonistic effects surmounted by addition of Ca^{2+} 3 mM. The concentration of antagonist was chosen such that Ca^{2+} (3 mM) resulted in a contraction which was 35–65% of the maximal response of the tissue. Salicylate (5 mM) was added when the Ca^{2+}-induced contrac-

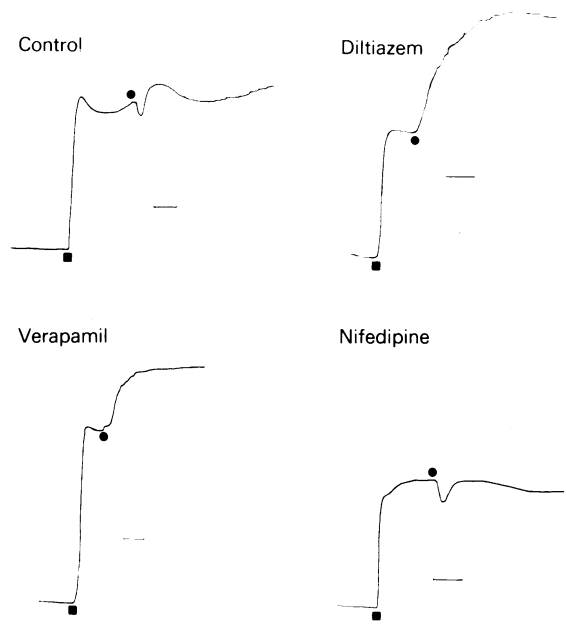

Figure 3 Effects of salicylate (5 mM, added at ●) on contractions induced by Ca^{2+} (3 mM, ■) of taenia preparations maintained in K^+ (40 mM)-depolarizing Tyrode solution. The preparations had been pretreated for 20 min before addition of Ca^{2+} with verapamil (0.2 μM), diltiazem (1 μM) or nifedipine (10 nM) The effects of salicylate on a contraction induced by Ca^{2+} (100 μM) in an untreated preparation are also shown (control). The time bar represents 10 min. The isotonic contractions were between 40 and 60% of the maximum contraction.

Table 3 Effects of salicylate (5 mM) on Ca^{2+}-induced contractions in K^+ depolarized taenia preparations pretreated with calcium-antagonists

	% change (s.e.mean)	n
Control	− 6.4 (8.7)	7
Verapamil, 0.2 μM	+ 63.8 (14.3)*	7
Diltiazem, 1 μM	- 86.2 (34.3)*	5
Nifedipine, 3 nM	+ 19.5 (12.0)	8
Cinnarizine, 1 μM	− 39.7 (15.1)*	8
Flunarizine, 1 μM	− 57.5 (13.3)*	8
Fendiline, 3 μM	− 64.6 (16.3)*	4
Pimozide, 1 μM	− 51.0 (17.2)*	5
Perhexiline, 10 μM	− 33.5 (9.9)*	4
W-7, 200 μM	− 98.0 (2.0)*	4

The experiments were performed as in Figures 3 and 4. * $P < 0.05$

tion had stabilized. Under these conditions salicylate caused a further increase in the Ca^{2+}-induced contraction when the preparations had been pretreated with verapamil (0.2 μM) or diltiazem (1 μM) (Figure 3, Table 3). Salicylate did not significantly change contractility in preparations pretreated with nifedipine (10 nM) (Figure 3, Table 3).

In contrast, salicylate (5 mM) caused a slow relaxation of Ca^{2+}-induced contractions in preparations which had been pretreated with cinnarizine (1 μM), flunarizine (1 μM), pimozide (1 μM), fendiline (3 μM), bepridil (3 μM) and perhexiline (10 μM) (Figure 4). Furthermore, salicylate (5 mM) fully relaxed Ca^{2+}-induced contractions when the taenia preparations had been preincubated with the calmodulin antagonist, W-7, 200 μM (Figure 4, Table 3).

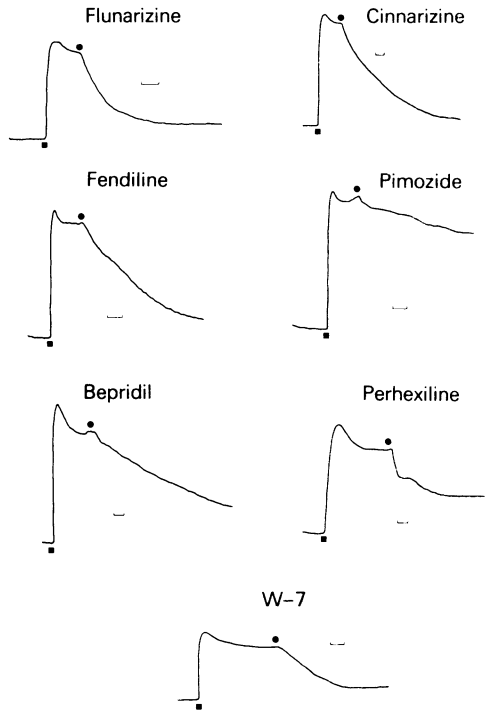

Figure 4 Effects of salicylate (5 mM, added at ●) on contractions induced by Ca^{2+} (3 mM, ■) of taenia preparations maintained in K^+ (40 mM)-depolarizing Tyrode solution. The preparations had been pretreated for 20 min before addition of Ca^{2+} with flunarizine (1 μM), cinnarizine (1 μM), fendiline (3 μM), pimozide (1 μM), bepridil (3 μM), perhexiline (10 μM) and W-7 (200 μM). The time bar represents 10 min. The isotonic contractions were between 40 and 60% of the maximum contraction.

Mechanism of salicylate-induced effects

Salicylate has been shown to change membranal surface charge at 5–10 mM, but may also inhibit prostaglandin biosynthesis or oxidative phosphorylation (Brasch, 1983). However, the differential effects of salicylate are unlikely to be due to inhibition of prostaglandin biosynthesis because indomethacin (28 μM) neither increased nor decreased Ca^{2+}-induced contractions in preparations pretreated with verapamil (0.2 μM) or cinnarizine (1 μM) ($n = 4$, $P > 0.1$). Furthermore inclusion of indomethacin (28 μM) in the K^+ Tyrode throughout the course of the experiment did not affect the ability of salicylate (5 mM) to augment a Ca^{2+}-induced contraction in a preparation pretreated with verapamil (0.2 μM) (data not illustrated).

The effects of sodium salicylate on the potency of the calcium antagonists were similarly not related to its well-known inhibitory effects on oxidative phosphorylation because 2,4-dinitrophenol did not change the potency of the calcium-antagonists (Table 4). The concentration of 2,4-dinitrophenol (20 μM) was chosen to cause threshold antagonism of Ca^{2+}-

Table 4 Effectiveness of calcium-antagonists in the absence or presence of 2,4-dinitrophenol (20 μM)

	Ca^{2+} dose-ratio (range s.e.mean)		
Antagonist	*Control*	*2,4-Dinitrophenol*	n
Verapamil, 0.2 μM	52.8 (35.0–90.2)	21 (7.1–42.9)	6
Cinnarizine, 1 μM	32.6 (14.0–75.9)	26.4 (13.2–52.7)	6

Experimental protocol as in Table 2.

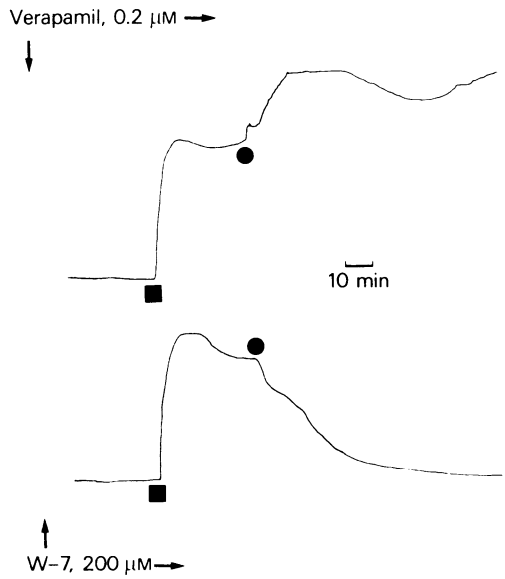

Figure 5 Effects of sodium benzoate (20 mM, ●) on contractions induced by Ca^{2+} (3 mM, ■) of taenia preparations maintained in K^+ (40 mM)-depolarizing Tyrode solution. The preparations had been pretreated with verapamil (0.2 μM for 20 min) or with W-7 (200 μM for 20 min). The isotonic contractions were 53 and 41% respectively of the maximum contraction.

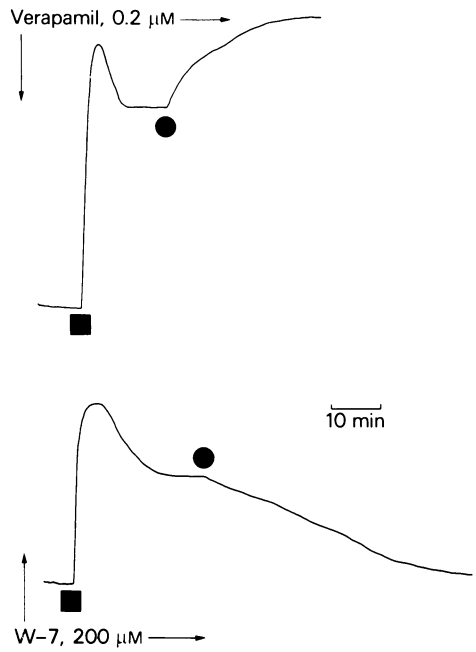

Figure 6 Effects of 3,5-dichlorosalicylate (100 μM, ●) on contractions induced by Ca^{2+} (3 mM, ■) of taenia preparations maintained in K^+ (40 mM)-depolarizing Tyrode solution. The preparations had been pretreated for 20 min before addition of Ca^{2+} with verapamil (0.2 μM) or with W-7 (200 μM). The isotonic contractions were 55 and 46% of the maximum contraction.

induced contractions; higher concentrations (55 μM) cause almost complete inhibition (Spedding, 1982a). Furthermore, 2,4-dinitrophenol (20 μM) did not augment Ca^{2+}-induced contractions in preparations pretreated with verapamil (0.2 μM, $n = 4$).

Sodium benzoate is a useful tool to investigate the effects of surface charge because although this agent is less potent than salicylate (Levitan & Barker, 1972) it does not uncouple oxidative phosphorylation (Brody, 1956; Brasch, 1983). Sodium benzoate (20 mM) resembled sodium salicylate in increasing Ca^{2+}-induced contractions in preparations pretreated with verapamil (0.2 μM) and relaxing preparations pretreated with W-7 (200 μM) (Figure 5). In contrast to sodium benzoate, 3,5-dichlorosalicylate is 50 fold more lipophilic than salicylate (Levitan & Barker, 1972) and is therefore adsorbed onto cell membranes at 30–100 fold lower concentrations (McLaughlin, 1973). 3,5-Dichlorosalicylate (100 μM) also increased Ca^{2+}-induced contractions in preparations treated with verapamil (0.2 μM) and relaxed contractions in preparations pretreated with W-7 (200 μM) (Figure 6). Thus the concentrations of sodium benzoate and of 3,5-dichlorosalicylate which have differential effects on calcium-antagonists are the concentrations previously found to change membrane surface charge.

If salicylate is acting by changing surface charge on the membrane then it may affect tissue K^+ permeability. Although the tissues were routinely maintained in high K^+ solution, the concentration used, 40 mM, is optimal for allowing reproducible and sustained Ca^{2+}-induced contractions but does not cause a maximal depolarization. Thus preparations were incubated with calcium-antagonists in K^+ (100 mM) Tyrode and challenged with salicylate (5 mM) after being contracted with Ca^{2+} (3 mM). Under these conditions the Ca^{2+}-induced contraction was not well maintained and, after an initial phasic contraction, declined to a steady level which was 30–70% less than the phasic contraction. Salicylate fully relaxed Ca^{2+}-induced contractions in preparations which had been pretreated with cinnarizine (1 μM) or W-7 (200 μM) ($n = 4$). However, salicylate (5 mM) had no effect in three preparations and relaxed one preparation (by 49%) which had been preincubated with verapamil, 0.2 μM. This result is in marked contrast with the findings in K^+ (40 mM)-Tyrode solution (Figure 3).

Local anaesthetics may inhibit Ca^{2+}-induced contractions in a non-specific fashion if sufficiently high

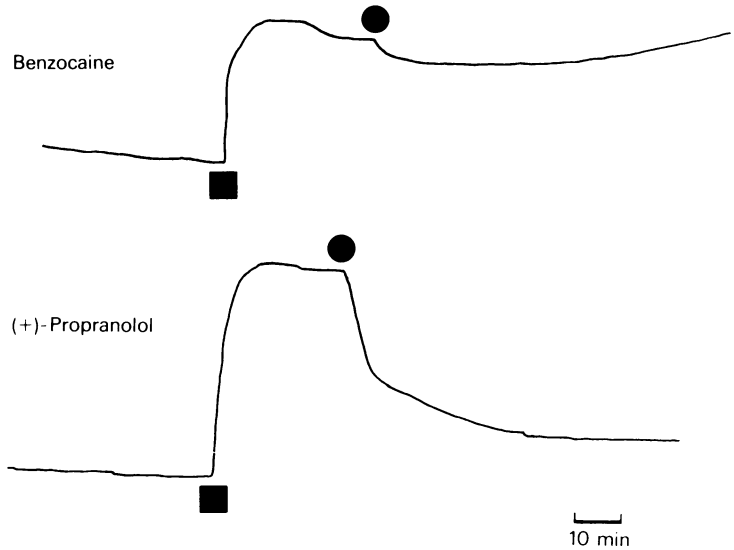

Figure 7 Effects of salicylate (5 mM, added at ●) on contractions induced by Ca^{2+} (3 mM, ■) of taenia preparations maintained in K^+ (40 mM)-depolarizing Tyrode solution. The preparations had been pretreated for 20 min before addition of Ca^{2+} with benzocaine (1 mM, upper panel) and (+)-propranolol (100 μM, lower panel). The isotonic contractions were 39 and 48% of the maximum contraction.

concentrations are used (Spedding & Berg, 1984). The effects of the neutral local anaesthetic benzocaine might be expected to be independent of surface charge. Taenia preparations were therefore incubated for 20 min with benzocaine (1 mM) or with the cationic local anaesthetic (+)-propranolol (100 μM). Salicylate (5 mM) caused a rapid relaxation of Ca^{2+}-induced contractions in (+)-propranolol-treated preparations but did not affect substantially the preparations pretreated with benzocaine (Figure 7).

Discussion

The drugs classed as calcium-antagonists are a chemically disparate group often with different clinical profiles. I have sought to rationalize these differences by proposing that three distinct subgroups of calcium antagonist exist, each with its own distinct structure activity relationships and different functional effects *in vitro* and *in vivo* (Spedding, 1981; 1982a,b; 1983). The present study has confirmed this classification because salicylate differentiated three subgroups of calcium-antagonists which were the same as those found in previous functional studies (Spedding, 1981; 1982a,b, 1983). Thus salicylate had no effect on the calcium-antagonist potency of the dihydropyridine, nifedipine, reduced the inhibitory effects of verapamil and diltiazem and increased the calcium-antagonistic effects of the lipophilic diphenylalkylamines (cinnarizine, flunarizine, fendiline, pimozide) and also of the calmodulin antagonist W-7. The effects of salicylate were similar whether preincubated with the antagonists or added to tissues where the calcium-antagonists were in a steady state with Ca^{2+}. Sodium salicylate thus appears to be a powerful tool to investigate the modes of action of different calcium-antagonists.

The mechanism of action of salicylate is probably due to an increase in negative surface charge of the membrane which would also affect the disposition of any residual bound Ca^{2+}. The concentrations of salicylate used are similar to those shown to change surface charge in artificial membranes (McLaughlin, 1973) and in molluscan neurones (Levitan & Barker, 1972). Furthermore, dichlorsalicylate was more potent and benzoate less potent than salicylate in causing similar effects, consistent with their differential affinities for lipid bilayers (McLaughlin, 1973) and neurones (Levitan & Barker, 1973). Further evidence for a role of surface charge is provided by the experiments with benzocaine and (+)-propranolol. Benzocaine is a neutral local anaesthetic which achieves its inhibitory effects by rapid diffusion into the membrane (Courtney, 1980). Predictably, the potency of this drug was not influenced by salicylate. In contrast the local anaesthetic properties of (+)-propranolol are predominantly due to the cationic form of the molecule. These effects should be influ-

enced by a change in surface charge and were in fact altered by salicylate. Furthermore, certain alternative explanations can be ruled out from the present work. For example, salicylate ions are known to uncouple oxidative phosphorylation (Brody, 1956; Brasch, 1983) and to inhibit prostaglandin biosynthesis. However, neither 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, nor indomethacin, an inhibitor of prostaglandin biosynthesis, had differential effects on calcium-antagonist potency. Nevertheless it is worth pointing out that these secondary effects will limit the test systems in which salicylate can be used because high concentrations (5 mM) are necessary to change surface charge; in heart preparations lower concentrations may inhibit oxidative phosphorylation and contractility (Brasch, 1983). It remains to be seen whether high concentrations of benzoate are more selective than salicylate in changing surface charge.

How may changes in surface charge differentially affect the action of calcium-antagonists? First, surface charge may affect ion channel gating and change the proportion of ion channels in a particular form (resting, activated, inactivated). Hille *et al.* (1975) proposed that negative surface charges clustered around sodium channels give a local contribution to the electrical field across the membrane and hence control gating characteristics. Shimoni (1981) has proposed that repriming of the Ca^{2+} channel in frog atrium is dependent on intracellular Ca^{2+} and on variations in extracellular surface charge. Thus the surface charge could influence the state of the channel and thereby alter the effectiveness of drugs which have affinity for a particular state.

Secondly, surface charges give a bulk charge to the membrane which can affect drug disposition. In this respect, penetration of cationic drugs in bilayer membranes is augmented by increases in negative surface charge (McLauglin, 1973). Surewicz & Leyko (1981) have shown that (+)-propranolol accumulates in and expands negatively charged acidic phospholipid membranes at low concentrations (5 μM), whereas 20 fold higher concentrations are required for an equivalent effect in neutral membranes. Thus the relaxation following addition of salicylate to propranolol-treated preparations may reflect greater partitioning of propranolol into the taenia muscle membranes following the increase in surface charge. In this respect, the time course of the relaxation following addition of salicylate is similar to the time course of the onset of the inhibitory effects of propranolol (Spedding & Berg, 1984, unpublished data), consistent with a change in equilibrium.

The increased effectiveness of W-7 and the diphenylalkylamines is also likely to be due to increased passage through cell membranes. W-7 binds to calmodulin (Hidaka *et al.*, 1978). Calmodulin functions as one of the Ca^{2+} receptor proteins for the contractile proteins in smooth muscle and W-7, in the concentrations used in this study (200 μM), has been shown to inhibit contractility in the taenia without inhibiting $^{45}Ca^{2+}$ uptake (Karaki *et al.*, 1982). Thus W-7 must penetrate the cell membrane to achieve its site of action and the increased effectiveness of W-7 in the presence of salicylate is probably due to increased intracellular accumulation, although a direct effect of salicylate on calmodulin cannot be ruled out. Similarly, the increased effectiveness of the diphenylalkylamines in the presence of salicylate may be taken as evidence that these drugs must accumulate at intracellular or intramembranal sites. An intracellular site of action on the contractile proteins has been reported (Spedding, 1983), but only at high concentrations requiring considerable intracellular accumulation. Intracellular accumulation has been demonstrated for one calcium-antagonist, bepridil, which binds to the contractile proteins (Cramb & Dow, 1983) and accumulates 40–100 fold in smooth or cardiac muscle (Cramb & Dow, 1983; Pang & Sperelakis, 1983). The diphenylalkylamines (Spedding, 1982a) and bepridil (unpublished observation) have a slow onset of action in the taenia which becomes even slower if Ca^{2+} is present in the bathing media. The reduced effectiveness of these drugs in the presence of high external Ca^{2+} and increased inhibition in the presence of salicylate is compatible with opposite effects of Ca^{2+} and salicylate on surface charge and hence on the disposition of the drugs.

The question then arises as to whether the drugs accumulate at the Ca^{2+} channel, at the contractile proteins, or at other sites. Fendiline and prenylamine have been shown to bind to calmodulin (Johnson *et al.*, 1983; Johnson & Fugman, 1983) at the same concentrations as they inhibit contractility in K^+-depolarized smooth muscle (Spedding, 1982a). Furthermore the other diphenylalkylamines, being lipophilic and weakly basic, would be expected to bind to the lipophilic site on this acidic protein (Spedding, 1983a). Thus, after accumulation the drugs could inhibit Ca^{2+}-dependent processes subsequent to Ca^{2+} entry. In this respect bepridil (Vogel *et al.*, 1979) and cinnarizine (Spedding & Richards, 1983) have been shown to inhibit contractility to a greater extent than $^{45}Ca^{2+}$ uptake (but see Godfraind, *et al.*, 1982). Nevertheless, the diphenylalkylamines do have high affinity for the calcium channel in smooth muscle, as defined by [^{3}H]-dihydropyridine binding (Bolger *et al.*, 1983). This apparent discrepancy can be resolved if the lipophilic recognition sites on the channel and on calmodulin are similar, as proposed by Johnson *et al.* (1983); the drugs would have affinity for both sites and, while the relative contribution at each site might depend on the experimental

situation, the end result in each case would be inhibition of contractility.

The reduced calcium-antagonistic effects of diltiazem and verapamil in the presence of salicylate may be due to direct effects on the calcium channel. Dichlorosalicylate (0.3 mM) reduces the inhibitory effects of verapamil, but not of dihydropyridines, on [^{3}H]-nitrendipine binding to guinea-pig skeletal muscle membranes (Mir & Spedding, unpublished observations.) A site of action at the calcium channel would therefore explain the reversal of the effects of verapamil by salicylate. However, such an explanation must also accomodate the finding that salicylate was only effective in reversing the effects of verapamil when the preparations were partly depolarized (40 mM K^+) and not when preparations were fully depolarized (100 mM K^+; Inomato & Kao, 1976) even though the effects of cinnarizine and W-7 were still augmented. This discrepancy might be explained on the basis that verapamil selectively binds to certain forms of the channel. Calcium channels may exist in at least three states (closed, open and inactivated; Reuter, 1983) and diltiazem and verapamil are claimed to bind selectively to the inactivated form (Kanaya *et al.*, 1983). Thus procedures reducing the time spent in this state would reduce the overall affinity of the channels for verapamil.

Shimoni (1981) has shown in frog atrium that increasing negative surface charge reduces and depolarization increases the proportion of inactivated channels which fits with the effects of salicylate in the taenia. It may therefore be possible that the effects of salicylate are overcome by maximal depolarization. Such a hypothesis would also explain the failure of salicylate to modify the effects of nifedipine, because the effects of this compound are much less dependent on the state of the channel (Kohlhardt & Fleckenstein, 1977; Bayer & Ehara, 1978).

The effects of diltiazem were also reduced by salicylate. It has been proposed that diltiazem dissociates a subunit from the calcium channel on the basis of target size analysis studies (Goll *et al.*, 1983); the effects of verapamil were not studied. As such subunits are probably held together by local charges, which may be modified *in vitro*, salicylate may be a useful tool for investigation of the function of the calcium channel in addition to studying the interactions of calcium antagonists with the channel.

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