

Direct activation of Ca²⁺ channels by palmitoyl carnitine, a putative endogenous ligand

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1 Palmitoyl carnitine, a lipid metabolite which accumulates in cytoplasmic membranes during ischaemia, has been shown to resemble the Ca²⁺ channel activator, Bay K 8644, in K⁺-depolarized smooth muscle. Palmitoyl carnitine caused concentration-dependent (1–1000 μmol l⁻¹) augmentations in the sensitivity to Ca²⁺ of K⁺-depolarized taenia preparations from the guinea-pig caecum. The (±)-isomer was equieffective with the (–)-isomer, whereas carnitine was ineffective and palmitic acid relaxed the tissues. The shift to the left of Ca²⁺ concentration-response curves induced by palmitoyl carnitine (100 μmol l⁻¹) was additive with that of Bay K 8644 (1 μmol l⁻¹).

2 The interactions of palmitoyl carnitine with the different classes of calcium-antagonist were similar to those seen with Bay K 8644. Schild plots of the calcium-antagonist effects of nifedipine were shifted to the right following preincubation of the taenia with palmitoyl carnitine (30–300 μmol l⁻¹). The inhibitory effects of verapamil were especially sensitive to palmitoyl carnitine (100 μmol l⁻¹). Whereas the potency of diltiazem as a calcium-antagonist was reduced by palmitoyl carnitine (100 μmol l⁻¹), the inhibitory effects of the lipophilic class III calcium-antagonists, cinnarizine and flunarizine, were entirely resistant to palmitoyl carnitine (100 μmol l⁻¹).

3 Although palmitoyl carnitine has detergent properties in high concentrations and lyses red blood cells, these effects were not Ca²⁺-dependent, nor were they modified by calcium-antagonists. Other detergents did not have selective interactions with Ca²⁺ channels.

4 Palmitoyl carnitine inhibited [³H]-nitrendipine, [³H]-verapamil and [³H]-diltiazem binding to rat cortical membranes with IC₅₀ values (μmol l⁻¹) of 120 ± 1, 95 ± 17 and 120 ± 15 μmol l⁻¹ respectively. The inhibition showed little temperature-dependence, in contrast to that of Bay K 8644, except for a small reduction in the IC₅₀ value for [³H]-verapamil binding at 37°C (42 ± 5 μmol l⁻¹). Palmitoyl carnitine interacted selectively with the Ca²⁺ channel, in that effects on ligand binding to α-adrenoceptors, β-adrenoceptors and 5-HT_{1A} receptors occurred only at 5–10 fold higher concentrations.

5 It is concluded that palmitoyl carnitine, at concentrations which have previously been shown to occur in the cytoplasm during myocardial ischaemia, may interact directly with Ca²⁺ channels and may therefore be considered as an endogenous modulator of channel function. The site of action differs from that of other agents.

Introduction

Increased free fatty acid levels have long been known to increase both susceptibility to arrhythmias and the severity of damage following ischaemia (Kurien & Oliver, 1966; deLeiris & Feuvray, 1977; Katz & Messineo, 1981; Neely & Feuvray, 1981; Corr *et al.*, 1984). Mitochondrial β-oxidation of fatty acids is blocked during ischaemia resulting in accumulation of acyl-CoA in mitochondria and of acyl carnitines in the cytosol (Idell-Wenger *et al.*, 1978; Liedtke *et al.*, 1978;

Neely & Feuvray, 1981; Knabb *et al.*, 1986). Acyl carnitines normally transport lipids into mitochondria but during ischaemia, cytosolic concentrations can reach up to 0.29 μmol g⁻¹ dry weight or 75–200 μmol l⁻¹ (Idell-Wenger *et al.*, 1978; Corr *et al.*, 1984).

Elegant work by Corr's group has shown that long chain acyl carnitines, such as palmitoyl carnitine, rapidly partition into the sarcolemma, and that hypoxia causes a 70 fold increase in sarcolemmal acyl carnitines with resultant electrophysiological disturbances (Knabb *et al.*, 1986). Previously, these distur-

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bances have been linked to the detergent properties of these amphipathic substances (Idell-Wenger *et al.*, 1978) and palmitoyl carnitine has been shown to exert many effects, such as inhibition of Na^+K^+ -ATPase (Adams *et al.*, 1979), Ca^{2+} -ATPase in myocardial sarcoplasmic reticulum (Pitts *et al.*, 1978) and biphasic effects on protein kinase C activation (Kato *et al.*, 1981; Wise *et al.*, 1982; Helfman *et al.*, 1983; Wise & Kuo, 1983). Nevertheless, Piper *et al.* (1984) concluded that the non-specific detergent effects of palmitoyl carnitine were insufficient to account for mitochondrial disruption following ischaemia. In this respect, we were interested by a report indicating that palmitoyl carnitine increased myocardial Ca^{2+} current, acting perhaps by a surface charge effect (Inoue & Pappano, 1983). Changes in surface charge modify Ca^{2+} channel function and markedly affect the susceptibility of the channels to calcium-antagonists (Spedding, 1984), presumably due to changes in channel state.

If palmitoyl carnitine were to activate Ca^{2+} channels, either directly or indirectly by a surface charge effect, then it may account for the deleterious Ca^{2+} overload which can occur following ischaemia, with reperfusion. We have therefore compared the effects of palmitoyl carnitine with those of Bay K 8644, a dihydropyridine Ca^{2+} channel activator (Schramm *et al.*, 1983) in K^+ -depolarized smooth muscle. The K^+ -depolarized taenia preparation from the guinea-pig caecum has been shown to be very sensitive to Ca^{2+} channel activators and antagonists (Spedding, 1982; Spedding & Berg, 1984) allowing quantitative analysis of interactions between the compounds (Spedding, 1985a). Analysis of the affinity of palmitoyl carnitine for the high affinity binding sites of ^3H -labelled calcium-antagonists was performed in rat cerebral cortex membranes, where affinity of agents for VOCs (voltage-operated Ca^{2+} channels) parallels inhibitory potency in smooth muscle. Some of these results have been published previously in abstract form (Mir & Spedding, 1986).

Methods

Smooth muscle

Taenia preparations from the caecum of male guinea-pigs (200–350 g) were set up in 10 ml isolated organ baths containing Ca^{2+} -free K^+ -Tyrode solution, of the following composition (mmol l^{-1}): NaCl 97, KCl 40, NaHCO_3 11.9, NaH_2PO_4 0.4, glucose 5.5. The solution was gassed with 95% O_2 :5% CO_2 and maintained at 35°C. Contractions were measured with Bioscience isotonic transducers, with 1 g load. Cumulative concentration-response curves for Ca^{2+} (0.1, 0.3, 1, 3, 10 mmol l^{-1}) were obtained by increasing the Ca^{2+}

concentration at 3–5 min intervals in logarithmic increments; the curves were obtained at 40 min intervals. The first Ca^{2+} concentration-response curve was only used to position the baseline and maximum response on the chart paper. Nevertheless the sensitivity of the first curve was similar to that of the second curve which was used to establish the control EC_{50} value. With this technique, the sensitivity to calcium does not change with time for 5 h (Spedding, 1982). Preparations were incubated with the antagonists for 25 min, in increasing concentrations. Ca^{2+} concentration-ratios are the $\text{Ca}^{2+} \text{EC}_{50}$ in the presence of the antagonist divided by the control EC_{50} . In parallel experiments in which the effects of the antagonists were assessed in the presence of palmitoyl carnitine, the antagonists were added 5 min after palmitoyl carnitine. Schild plots (Arunlakshana & Schild, 1959) were made by plotting $\log(\text{Ca}^{2+} \text{concentration-ratio} - 1)$ against $-\log$ of the molar concentration of the antagonist. The slopes of the Schild plots were calculated by linear regression analysis and compared by analysis of covariance (Winer, 1962).

The Ca^{2+} concentrations quoted in the text refer to CaCl_2 added to the medium. The low phosphate Tyrode solution used was designed for maximum Ca^{2+} solubility without the use of artificial buffers such as HEPES, which reduce the number of reproducible concentration-response curves obtainable (Spedding, unpublished). The Ca^{2+} concentration was monitored in some experiments with a Ca^{2+} electrode (Philips) and the Ca^{2+} concentrations were not significantly ($P > 0.1$) lower in the bicarbonate/phosphate buffer than in distilled water. Ca^{2+} in the distilled water was $< 2 \mu\text{mol l}^{-1}$. Nevertheless, the Ca^{2+} concentration in the centre of the muscle bundles could be expected to be higher than in the bathing medium.

The dihydropyridines (1 mmol l^{-1}) were dissolved in ethanol before dilution in distilled water. The highest concentration of ethanol which was used (0.1%) did not cause significant antagonistic effects (Ca^{2+} concentration-ratio < 2). The solutions were protected from light. All the concentrations refer to the final bath concentrations of the drugs. Mean values are quoted \pm s.e.mean.

Rat cortical membranes

Male Sprague-Dawley rats (250–350 g) were killed by decapitation and the brains quickly removed. Cerebral cortex was dissected from the rest of the brain over ice and stored at -70°C . Membranes were freshly prepared each day by homogenizing the tissue in ice-cold Tris-HCl buffer (50 mmol l^{-1} , pH 7.4) at a concentration of $30 \text{ mg tissue ml}^{-1}$ with a Kinematica polytron homogenizer (10s, setting 3). The homogenate was centrifuged at $4800 g$ for 10 min and the pellet subsequently washed twice ($4800 g \times 10 \text{ min}$) with

fresh buffer.

For competition experiments with [³H]-nitrendipine, aliquots (200 μl) of the tissue homogenate (100 mg 3 ml⁻¹) were incubated in duplicate with 0.1 nmol l⁻¹ [³H]-nitrendipine. Incubations were carried out under subdued light for 90 min at 25°C in Tris-HCl buffer (50 mmol l⁻¹, pH 7.4) in a final volume of 1 ml. Incubations were terminated by addition of 3 ml ice-cold buffer and membrane-bound and free ligand were separated by rapid filtration over Whatman GF/C filters with a modified Brandel cell harvester; they were subsequently washed with ice-cold Tris buffer (20 mmol l⁻¹, pH 7.4, MgCl₂, 10 mmol l⁻¹, 10% polyethylene glycol, 2 × 5 ml). Radioactivity retained on the filters was measured by liquid scintillation spectrophotometry with a counting efficiency of approximately 42%. Non-specific binding was determined in the presence of nifedipine 0.1 μmol l⁻¹ and specific binding represented about 85% of the total binding.

For [³H]-diltiazem, binding aliquots (400 μl) of the tissue homogenate (200 mg 3 ml⁻¹) were incubated with 2.5–3.0 nmol l⁻¹ [³H]-diltiazem in Tris-HCl buffer (50 mmol l⁻¹, pH 7.4) in a final volume of 1 ml at 25°C and 37°C for 90 min and 60 min, respectively. Membrane bound radioactivity was collected by rapid filtration over Whatman GF/B filters and washed with ice-cold Tris-HCl buffer. Specific binding was defined as the binding inhibited by unlabelled (+)-*cis*-diltiazem (10 μmol l⁻¹) and represented about 70% and 58% of the total binding at 25°C and 37°C respectively. To reduce the radioactivity attaching to the filters, the filters were pretreated with 0.05% polyethylenimine and under these washing conditions, the filter blank radioactivity was reduced and was not displaceable by unlabelled diltiazem.

For [³H]-verapamil binding, aliquots (400 μl) of the tissue homogenate (100 mg 3 ml⁻¹) were incubated with 1.5–2.0 nmol l⁻¹ [³H]-verapamil in Tris-HCl buffer (50 mmol l⁻¹, pH 7.4) in a volume of 1 ml at 25°C and 37°C for 90 min and 60 min, respectively. Washing procedures were the same as described above and the filters were washed with 0.05% polyethylenimine before the start of filtration, to reduce the binding of radioactivity to the filters. Specific [³H]-verapamil binding was defined as the binding displaced by unlabelled (±)-verapamil (10 μmol l⁻¹) and represented 70–75% of the total binding. Results are expressed as mean values ± s.e. of at least 3 separate experiments.

Affinity of palmitoyl carnitine for α₁- and β-adrenoceptors and for 5-HT_{1A} receptors in cortical membranes was assessed by use of [³H]-prazosin, [³H]-dihydroalprenolol, [³H]-8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) as described by Palfreyman *et al.* (1986).

Results

Effects in smooth muscle

Taenia preparations in K⁺-depolarizing Tyrode solution relaxed fully in Ca²⁺-free medium. Contractions in response to Ca²⁺ (0.1–3 mmol l⁻¹) were reproducible and stable for long periods of time (up to 5 h). Palmitoyl carnitine (100 μmol l⁻¹) and Bay K 8644 (1 μmol l⁻¹) augmented established submaximal contractions in response to Ca²⁺ (100 μmol l⁻¹; Figure 1). Furthermore, the preparations relaxed rapidly when the exogenous Ca²⁺ was chelated with EDTA (150 μmol l⁻¹), indicating that neither palmitoyl carnitine nor Bay K 8644 interfered with the mechanisms involved in relaxation, consistent with an effect involving facilitated Ca²⁺ entry into the smooth muscle cells. Following washout of the drugs from the baths, the tissues showed a continued slight increase in sensitivity to Ca²⁺ (Figure 1) indicating that the drugs washed out slowly from the tissues and that palmitoyl carnitine did not adversely affect contractility.

The concentration-response curve for palmitoyl carnitine, in increasing Ca²⁺-induced contractions is shown in Figure 2. The effects of palmitoyl(-)-carnitine were not significantly different from those of palmitoyl(±)-carnitine, which was used in all further studies. The EC₅₀ for palmitoyl carnitine was 75 ± 5 μmol l⁻¹. Carnitine was inactive, whereas palmitic acid relaxed Ca²⁺-induced contractions, albeit at higher concentrations (Figure 2). Lysophosphatidyl choline was more than 10 fold less effective than

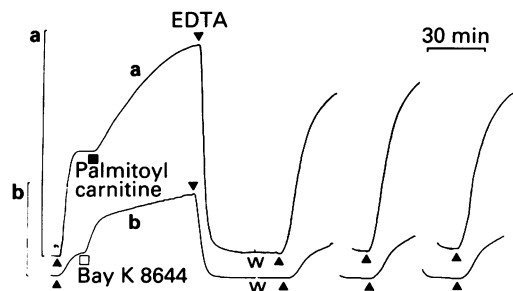


Figure 1 Comparison of the effects of palmitoyl carnitine (100 μmol l⁻¹, ■, a) and Bay K 8644 (1 μmol l⁻¹, □, b) on Ca²⁺-induced contractions (▲, 100 μmol l⁻¹) in K⁺-depolarized taenia preparations from the guinea-pig caecum. EDTA (▼, 150 μmol l⁻¹) was added to chelate bath Ca²⁺; the preparations relaxed rapidly indicating that relaxant mechanisms were not impaired by the compounds. After washout (W) the sensitivity of the preparations remained high to Ca²⁺. Vertical calibration bars represent the maximum response to Ca²⁺ (3 mmol l⁻¹).

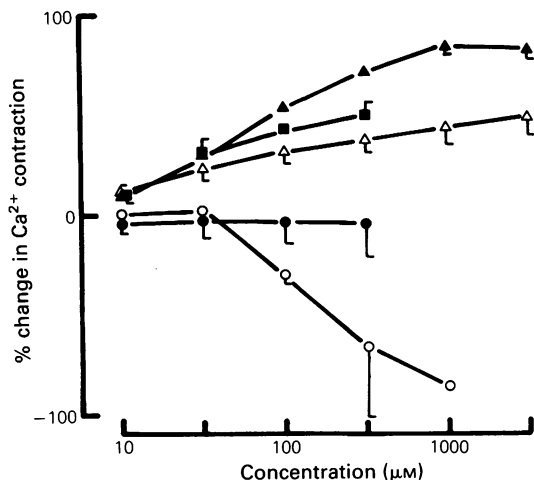


Figure 2 Effects of palmitoyl(±)-carnitine (▲), palmitoyl(-)-carnitine (■), carnitine (●) and palmitic acid (○) on submaximal contractions induced by Ca^{2+} ($100 \mu\text{mol l}^{-1}$) in K^+ -depolarized taenia preparations from the guinea-pig caecum. In one series of experiments preparations were pretreated with Bay K 8644 ($1 \mu\text{mol l}^{-1}$, Δ) and the preparations submaximally contracted with Ca^{2+} ($30 \mu\text{mol l}^{-1}$); in these preparations palmitoyl carnitine further increased the Ca^{2+} -induced contractions.

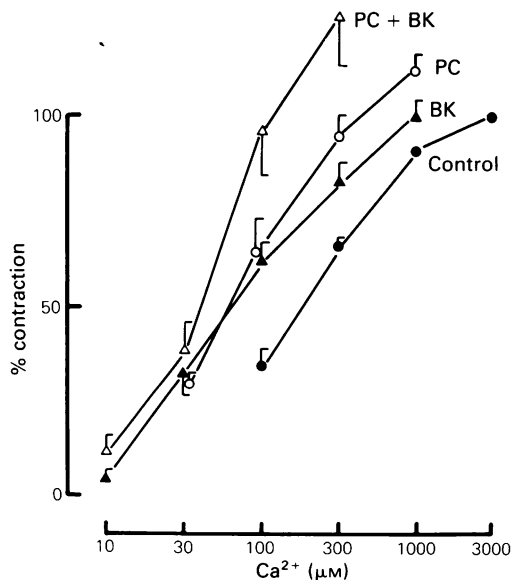


Figure 3 Shift to the left of Ca^{2+} concentration-response curves (● control) in K^+ -depolarized taenia preparations by palmitoyl carnitine (PC, $100 \mu\text{mol l}^{-1}$, ○), Bay K 8644 (BK, $1 \mu\text{mol l}^{-1}$) or a combination of the two agents (PC + BK, Δ). Vertical bars represent s.e.mean of 5 experiments.

palmitoyl carnitine (not shown). Palmitoyl carnitine was capable of increasing Ca^{2+} -induced contractions in the presence of a maximally-effective concentration of Bay K 8644 (Figure 2). Thus, when taenia preparations were incubated with Bay K 8644, using a concentration ($1 \mu\text{mol l}^{-1}$) previously shown to be maximally effective (Spedding & Berg, 1984), and submaximally contracted with Ca^{2+} ($30 \mu\text{mol l}^{-1}$), palmitoyl carnitine caused a further increase in the Ca^{2+} contraction (Figure 1); Bay K 8644 ($1-10 \mu\text{mol l}^{-1}$) did not augment a Ca^{2+} -induced contraction under these conditions.

Palmitoyl carnitine ($100 \mu\text{mol l}^{-1}$) shifted concentration-response curves to Ca^{2+} to the left, as did Bay K 8644 ($1 \mu\text{mol l}^{-1}$; Figure 3). Combination of the two agents caused a further shift to the left of the Ca^{2+} concentration-response curves.

Interactions with calcium-antagonists

Bay K 8644 has been shown to have distinct, selective interactions with different subgroups of calcium-antagonists (Spedding, 1985b) and a similar protocol was used in the studies with palmitoyl carnitine. Nifedipine has been shown to reduce the sensitivity to Ca^{2+} in K^+ -depolarized taenia in a pseudo-com-

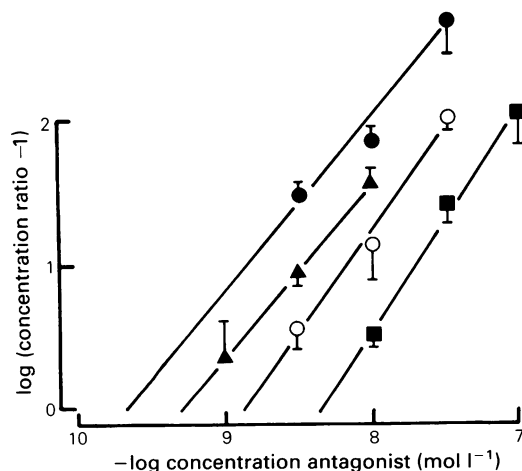


Figure 4 Schild plot of the inhibitory effects of nifedipine in K^+ -depolarized taenia preparations in the absence (●) or presence of palmitoyl carnitine ($30 \mu\text{mol l}^{-1}$ ▲; $100 \mu\text{mol l}^{-1}$ ○; $300 \mu\text{mol l}^{-1}$ ■). Nifedipine was preincubated, in increasing concentrations, for 25 min prior to cumulative addition of Ca^{2+} ($0.1-10 \mu\text{mol l}^{-1}$). Palmitoyl carnitine was added 5 min before nifedipine. The apparent pA_2 values are listed in Table 1; the slopes of the Schild plots were all 1.2 ± 0.2 , indicating a parallel shift to the right. Vertical lines represent s.e.mean, $n = 5$.

petitive manner (Spedding, 1982; 1985b), as the drug shifted Ca²⁺ concentration-response curves to the right, in parallel. In the present experiments, nifedipine was found to have an apparent pA₂ as an antagonist of Ca²⁺-induced contractions of 9.7 ± 0.1 (slope 1.2 ± 0.2; Figure 4). Preincubation with palmitoyl carnitine (30–300 μmol l⁻¹) increased the sensitivity of the taenia to Ca²⁺ and reduced the potency of nifedipine as a calcium-antagonist (Figure 4), shifting the Schild plots to the right, in parallel (Table 1). Bay K 8644 (10–1000 nmol l⁻¹) has similar effects (Table 1).

The calcium-antagonistic effects of diltiazem were also reduced by palmitoyl carnitine (100 μmol l⁻¹), in that the Schild plot was displaced to the right, in parallel (control slope 1.0 ± 0.1; palmitoyl carnitine 1.0 ± 0.1) and the apparent pA₂ reduced (Table 1).

Palmitoyl carnitine (100 μmol l⁻¹) caused very marked antagonism of the inhibitory effects of verapamil with non-parallel depression of the Schild plots (control slope 1.2 ± 0.3; palmitoyl carnitine slope 0.5 ± 0.3) and a reduction of the apparent pA₂ (Table 1).

In contrast, the inhibitory effects of flunarizine and cinnarizine, lipophilic class III calcium-antagonists (Spedding, 1985a), were resistant to palmitoyl carnitine (100 μmol l⁻¹) (Table 1). In this respect, palmitoyl carnitine resembled Bay K 8644 which does not reverse the inhibitory effects of this class of calcium-antagonist (Table 1).

Detergent effects of palmitoyl carnitine

In high concentrations, acyl carnitines may act as detergents and disrupt cell membranes. In this respect, palmitoyl carnitine (100 μmol l⁻¹) caused submaximal lysis of canine erythrocytes (Figure 5). However, the

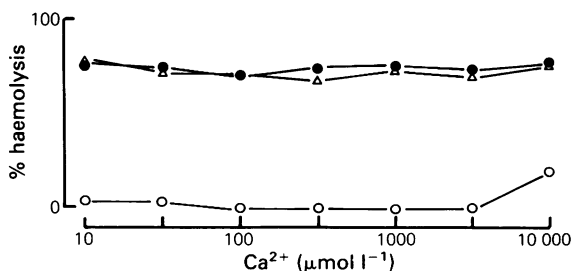


Figure 5 Lysis of red blood cells by palmitoyl carnitine (100 μmol l⁻¹) in the presence (Δ) or absence (●) of verapamil (10 μmol l⁻¹); 50 μl of dog packed red blood cells were added to 10 ml tubes containing saline and various Ca²⁺ concentrations. In some experiments palmitoyl carnitine was not added (○). Note that palmitoyl carnitine caused 80% lysis and that this was unaffected by the Ca²⁺ concentration or by verapamil.

Table 1 Apparent pA₂ values for calcium-antagonists in the presence of palmitoyl carnitine, Bay K 8644, sodium dodecyl sulphate or saponin

Calcium antagonist	Nifedipine	Diltiazem	Verapamil	Cinnarizine	Flunarizine
Control ^a	9.3 ± 0.3 (9)	7.8 ± 0.2 (7)	7.8 ± 0.2 (7)	7.9 ± 0.1 (7)	7.8 ± 0.2 (5)
+ Bay K 8644					
10 nmol l ⁻¹	8.4 ± 0.2 (7)*				
100 nmol l ⁻¹	7.7 ± 0.2 (7)***				
1000 nmol l ⁻¹	6.7 ± 0.2 (7)***	7.1 ± 0.2 (7)**	6.1 ± 0.4 (7)***	7.9 ± 0.1 (7)	7.8 ± 0.2 (5)
Control	9.7 ± 0.2 (5)	7.7 ± 0.1 (5)	8.3 ± 0.3 (5)	7.5 ± 0.2 (5)	7.4 ± 0.2 (5)
+ palmitoyl carnitine					
30 μmol l ⁻¹	9.4 ± 0.2 (5)*				
100 μmol l ⁻¹	8.9 ± 0.1 (5)***	7.0 ± 0.1 (5)***	7.6 ± 0.3 (5)**	7.3 ± 0.1 (5)	7.2 ± 0.2 (5)
300 μmol l ⁻¹	8.3 ± 0.1 (5)***				
Control	9.7 ± 0.2 (5)			7.7 ± 0.2 (5)	
+ sodium dodecyl sulphate					
10 μmol l ⁻¹	9.5 ± 0.2 (5)				
100 μmol l ⁻¹	9.3 ± 0.2 (5)				
Control	9.7 ± 0.2 (5)				
+ saponin					
100 μg ml	9.8 ± 0.1 (5)			7.7 ± 0.3 (5)	

^aData from Spedding (1985a) or Spedding & Berg (1984) using the same protocol. *P < 0.05; **P < 0.01; ***P < 0.001.

lysis was independent of the Ca^{2+} concentration and was unaffected by verapamil ($10 \mu\text{mol l}^{-1}$; Figure 5), or by nifedipine ($1 \mu\text{mol l}^{-1}$) or cinnarizine ($1 \mu\text{mol l}^{-1}$; not shown). The degree of lysis was highly dependent on the volume of erythrocytes added, in that doubling the volume from $50 \mu\text{l}$ to $100 \mu\text{l}$ reduced the degree of lysis from 80% to 20%. Other surface-active agents which lyse erythrocytes did not resemble palmitoyl carnitine in K^{+} -depolarized smooth muscle (Figure 6). Triton X-100, Tween 80 and saponin relaxed Ca^{2+} -induced contractions; saponin did not antagonize the effects of nifedipine (Table 1). Although sodium dodecyl sulphate augmented Ca^{2+} -induced contractions, this agent did not reduce the potency of nifedipine.

Interactions with ^3H -ligands

Binding sites within the Ca^{2+} channel for dihydropyridines, diltiazem and verapamil are allosterically linked and the interactions between the sites are temperature-dependent, allowing analysis of the site of action. [^3H]-nitrendipine bound with high affinity to a single site in guinea-pig cortical membranes, as previously described (Mir & Spedding, 1987). Nitrendipine, Bay K 8644 and palmitoyl car-

nitine and palmitic acid displaced [^3H]-nitrendipine binding, but carnitine was ineffective (Figure 7). The IC_{50} values are shown in Table 2; the IC_{50} for palmitoyl carnitine was similar to that required to augment Ca^{2+} -induced contractions in K^{+} -depolarized smooth muscle.

The effects of dihydropyridines on [^3H]-diltiazem binding are very temperature-sensitive and nitrendipine and Bay K 8644 were found to increase specific [^3H]-diltiazem binding at 37°C but to inhibit binding at 25°C (Figure 8). In contrast, palmitoyl carnitine inhibited [^3H]-diltiazem binding at both 25°C and 37°C . The EC_{50} and IC_{50} values are listed in Table 2.

The dihydropyridines inhibited only a small proportion of specific [^3H]-verapamil binding, the proportion of which is essentially unaffected by temperature (Figure 9). Palmitoyl carnitine resembled verapamil in that both agents caused a similar maximal displacement of [^3H]-verapamil binding (Figure 9). Furthermore, palmitoyl carnitine was more potent in displacing [^3H]-verapamil binding than [^3H]-nitrendipine or [^3H]-diltiazem binding (Table 2).

Palmitoyl carnitine selectively interacted with ligands binding to the Ca^{2+} channel, because the binding of agents at α -adrenoceptors, β -adrenoceptors and 5-HT_{1A} receptors was inhibited only by concentra-

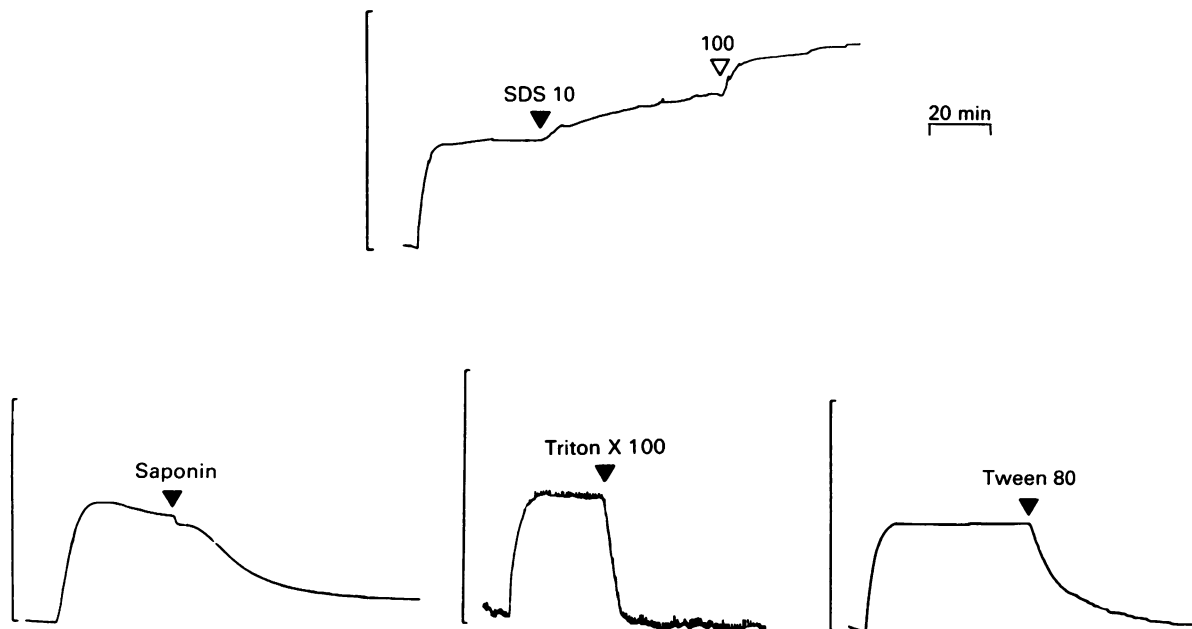


Figure 6 Effects of sodium dodecyl sulphate (SDS, 10 and $100 \mu\text{mol l}^{-1}$), saponin ($100 \mu\text{g ml}^{-1}$), Triton X 100 (0.3%) and Tween 80 (0.3%) on submaximal contractions of K^{+} -depolarized taenia preparations induced by Ca^{2+} ($100 \mu\text{mol l}^{-1}$). The vertical bars represent the maximal contractions of the tissues.

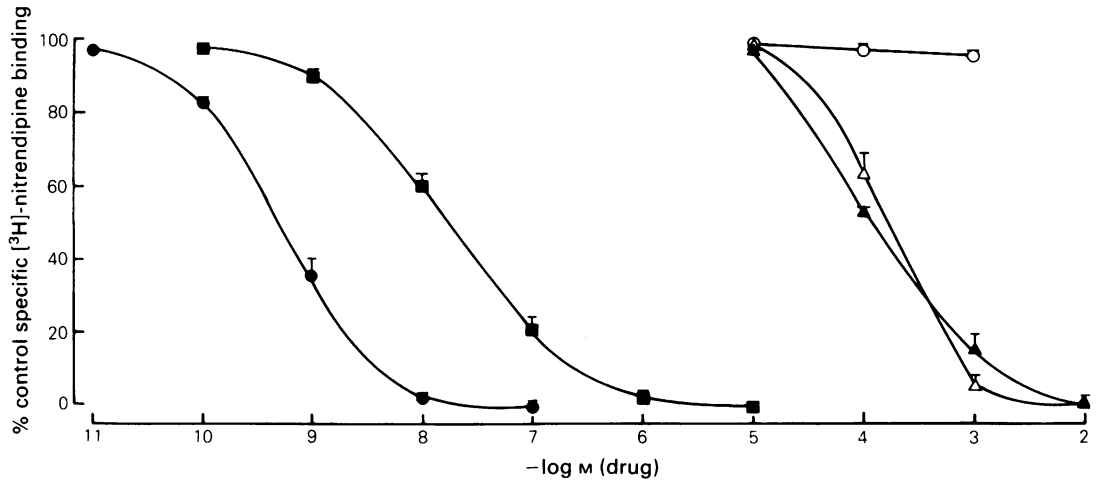


Figure 7 Effects of palmitoyl carnitine (▲), palmitic acid (△), carnitine (○), nitrendipine (●) and Bay K 8644 (■) on [³H]-nitrendipine binding to rat cerebral cortex membranes, at 25°C. Vertical lines represent s.e.mean, *n* = 3–7.

Table 2 Comparison of the potencies of nitrendipine, Bay K 8644 and palmitoyl carnitine in affecting the binding of radioligands to rat cerebral cortex membranes

	IC ₅₀ or EC ₅₀ , μmol l ⁻¹ ± s.e.mean (<i>n</i>)	
	25°C	37°C
[³H]-nitrendipine binding		
Nitrendipine	0.0006 ± 0.0001 (4)	—
Bay K 8644	0.022 ± 0.005 (7)	—
Palmitoyl carnitine	120 ± 1.06 (3)	—
Palmitic acid	170 ± 1.06 (3)	—
[³H]-diltiazem binding		
Nitrendipine	0.0013 ± 0.0003 (3)	0.0004 ± 0.0001 (3)
Bay K 8644	0.0133 ± 0.0002 (3)	0.017 ± 0.005 (3)
Palmitoyl carnitine	120 ± 15 (4)	173 ± 17 (4)
[³H]-verapamil binding		
Nitrendipine	0.00085 ± 0.0015 (5)	0.00142 ± 0.000056 (4)
Bay K 8644	0.0071 ± 0.0036 (5)	0.038 ± 0.005 (4)
Verapamil	0.057 ± 0.012 (5)	0.116 ± 0.017 (4)
Palmitoyl carnitine	95 ± 17 (4)	42 ± 5 (4)
[³H]-prazosin binding		
Palmitoyl carnitine	437 ± 60 (4)	
[³H]-8-OH-DPAT binding		
Palmitoyl carnitine	447 ± 60 (3)	
[³H]-dihydroalprenolol binding		
Palmitoyl carnitine	398 ± 46 (4)	

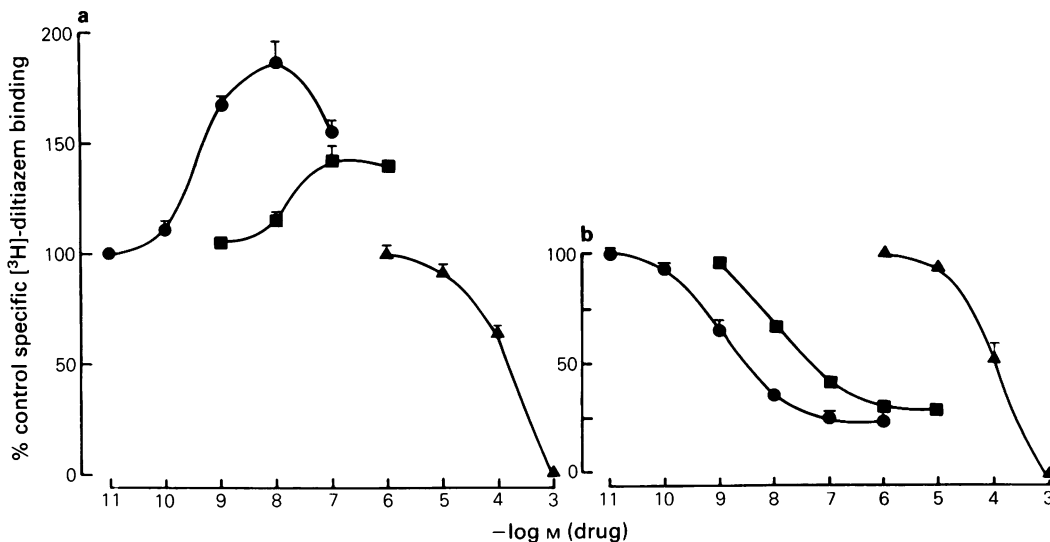


Figure 8 Effects of palmitoyl carnitine (▲), nitrendipine (●) and Bay K 8644 (■) on [³H]-diltiazem binding to rat cerebral cortex membranes at 37°C (a) or 25°C (b). Vertical lines represent s.e.mean, *n* = 3–4.

tions of palmitoyl carnitine that were much higher (Table 2).

Discussion

Receptor sites may be located directly on ion channels, as is the case for the nicotinic receptor (Hille, 1984). VOCs have been shown to have high affinity receptor sites for calcium-antagonists, yet no endogenous ligand has been shown to interact directly with the Ca²⁺ channel, as yet. The predominant control of

VOC function is by changes in membranal polarization (Reuter, 1967) or surface charge (Spedding, 1984; Hille, 1984), with further indirect modulation occurring via channel phosphorylation (heart; cyclic AMP dependent protein kinase, Tsien *et al.*, 1972; Speralakis & Schnieder, 1976; Brum *et al.*, 1983; neurones: protein kinase C, Harris *et al.*, 1986; Messing *et al.*, 1986; Osugi *et al.*, 1986; Wakade *et al.*, 1986) or interactions with G proteins (Holz *et al.*, 1986). We were therefore interested by the report of Inoue & Pappano (1983) indicating that palmitoyl carnitine increased Ca²⁺ current in avian ventricular

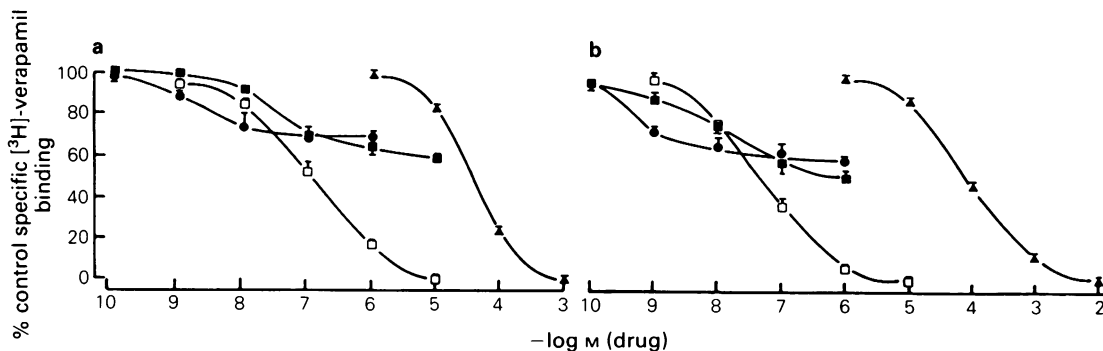


Figure 9 Effects of palmitoyl carnitine (▲), nitrendipine (●), Bay K 8644 (■) and verapamil (□) on [³H]-verapamil binding to rat cerebral cortex membranes at 37°C (a) and 25°C (b). Vertical lines represent s.e.mean, *n* = 4–5.

muscle; palmitoyl carnitine might therefore be considered as a potential endogenous ligand.

Palmitoyl carnitine resembled Bay K 8644, in that it increased the sensitivity of smooth muscle to Ca²⁺, under depolarizing conditions, without impairing the ability of the tissue to relax, findings which are consistent with selective activation of VOCs. Palmitoyl carnitine and Bay K 8644 had additive effects, which might point to different molecular sites of action in the Ca²⁺ channel. However, this difference might be secondary to the fact that Bay K 8644 is not a full activator and has a bell-shaped concentration-response curve (Spedding & Berg, 1984; Thomas *et al.*, 1984; Spedding *et al.*, 1987). Thus the additive effect of palmitoyl carnitine with Bay K 8644 might be consequent upon high concentrations of palmitoyl carnitine having less antagonistic effects at VOCs than Bay K 8644.

Bay K 8644, by virtue of its affinity for the dihydropyridine site, has selective interactions with the different classes of calcium-antagonists in smooth muscle (Spedding, 1985a,b). Under constant depolarizing conditions, the calcium-antagonistic effects of other dihydropyridines such as nifedipine are competitively antagonized by Bay K 8644 (Su *et al.*, 1984; Spedding, 1985a). The effects of the class II antagonists such as verapamil and diltiazem are reversed non-competitively (Schramm & Towart, 1984; Su *et al.*, 1984; Spedding, 1985a), which is consistent with the allosteric linkage between the binding sites for these drugs in the Ca²⁺ channel (Janis & Triggle, 1984; Glossmann *et al.*, 1986). In complete contrast, Bay K 8644 does not reverse the inhibitory effects of class III calcium-antagonists such as cinnarizine or flunarizine (Spedding & Berg, 1984); this discrepancy is consequent upon these agents having a molecular site of action which may either be in VOCs (Godfraind & Miller, 1983), trapping the channels in a state which has low affinity for dihydropyridines, or be at other sites (Spedding, 1985b; Grima *et al.*, 1986). Identical interactions occur in the heart (Boddeke *et al.*, 1987) and may be considered as a powerful tool to dissect the molecular site of action of an activator or an antagonist at VOCs.

Palmitoyl carnitine also had selective interactions with the different calcium-antagonists. First, the calcium-antagonistic effects of nifedipine were antagonized, although the extent of the antagonism was not as great as with Bay K 8644, presumably because palmitoyl carnitine is not selective for the dihydropyridine site. Secondly, the reversal of the effects of verapamil and diltiazem was very similar to that seen with Bay K 8644, even to the extent that the Schild plot for diltiazem was displaced in a parallel manner by both substances whereas the plot for verapamil was flattened (see Spedding, 1985a). Thirdly, palmitoyl carnitine, like Bay K 8644, was ineffec-

tive in reversing the calcium-antagonist effects of cinnarizine and flunarizine, which is powerful evidence for palmitoyl carnitine having a site of action at VOCs.

These findings are supported by the ligand binding studies where the concentrations of palmitoyl carnitine required to displace [³H]-nitrendipine, [³H]-diltiazem and [³H]-verapamil binding were similar to those required to reduce the calcium-antagonistic effects of these drugs. Palmitoyl carnitine was selective for agents binding to the Ca channel. It is likely that palmitoyl carnitine may interact with the binding site for verapamil. [³H]-verapamil binding was most susceptible to palmitoyl carnitine, and the calcium-antagonist effect of verapamil were also very susceptible. Palmitoyl carnitine, unlike the dihydropyridines, displaced all the specific [³H]-verapamil binding. Furthermore, verapamil reduces [³H]-nitrendipine binding and [³H]-diltiazem binding in rat cortical membranes in a manner which is not markedly temperature-dependent (Mir & Spedding, 1986), like the effects of palmitoyl carnitine. Nevertheless, although these findings point to a direct interaction at the verapamil binding site, certain reservations must be made. Palmitoyl carnitine is a lipophilic amphipathic substance which will incorporate into cell membranes and change a variety of functional parameters (Bigard & Spedding, 1986), including Ca²⁺ binding. The verapamil site is particularly sensitive to changes in surface charge (Spedding, 1984), membrane depolarization (Linden & Brooker, 1980; Spedding *et al.*, 1986) and to cytosolic Ca²⁺ (Reynolds *et al.*, 1983; Galizzi *et al.*, 1985) and this protein may be the voltage sensor for the channel. It is therefore probable that the changes induced by palmitoyl carnitine in the binding and in the calcium-antagonistic potency of verapamil are consequent upon a direct interaction with the Ca²⁺ channel or tightly associated lipids, but may not necessarily be due to direct competition with verapamil for its site of action.

It is unlikely that the effects of palmitoyl carnitine can be related simply to its detergent effects, even though these overlap the higher end (0.1–1 mmol l⁻¹) of the concentration-range we have used. The degree of lysis of erythrocytes was totally dependent on the volume of erythrocytes added, indicating that accumulation into the cell membranes is a crucial factor. However, we observed no significant change in the EC₅₀ for augmentation of Ca²⁺-induced contractions between small (<5 mg) or large (6–10 mg) taenia preparations (data not shown). Only sodium dodecyl sulphate of the detergents tested increased the sensitivity of the taenia to Ca²⁺ and this agent did not reduce the sensitivity of the preparations to nifedipine. The increased Ca²⁺ sensitivity of the tissues presumably reflects the affinity of sodium dodecyl sulphate for the Na⁺/Ca²⁺ exchanger (Philipson, 1984; Philipson *et*

al., 1985) and not affinity for VOCs. Palmitoyl CoA and lysophosphatidyl choline have similar, or greater potency to lyse erythrocytes compared with palmitoyl carnitine (Brecher, 1983; Eskelinen & Mela, 1984; unpublished observations) yet both agents were considerably less potent than palmitoyl carnitine in enhancing Ca²⁺-induced contractions in the taenia. Both agents cause electrophysiological disturbances in the heart, with some augmentation of Ca²⁺ current (Corr *et al.*, 1981; 1982), but the present findings indicate that palmitoyl carnitine is more selective for the Ca²⁺ channel, at least under constant depolarizing conditions where effects on outward currents will be minimized.

Nevertheless, these findings should not be taken as an indication that the effects of palmitoyl carnitine on VOCs are independent of the detergent properties of the molecule. Adams *et al.* (1979) considered palmitoyl carnitine to be a 'naturally occurring detergent' with biphasic effects on a variety of ion transport systems, following incorporation into the membrane. We found decanoylcarnitine to be considerably less effective than palmitoyl carnitine in causing lysis of erythrocytes and in augmenting Ca²⁺-induced contractions. It is likely that the VOCs may be particularly sensitive to the physico-chemical properties of palmitoyl carnitine and in this respect the calcium-antagonist verapamil has been considered to exert its use-dependent effects by relatively simple physico-chemical interactions with a subunit of the Ca²⁺ channel (Mannhold *et al.*, 1984). Selective 'detergent-like' effects of palmitoyl carnitine within VOCs may therefore explain the selective interactions seen in smooth muscle.

As acyl carnitines have been shown to accumulate in high concentrations in the myocardium, and to

increase Ca²⁺ current (Corr *et al.*, 1981; 1982; Inoue & Pappano, 1983), to exert positive inotropic effects (Inoue & Pappano, 1983; Duncan *et al.*, 1986) and to reverse the inhibitory effects of nisoldipine and verapamil (Duncan *et al.*, 1986), they may therefore be considered as endogenous Ca²⁺ channel activators. Any selectivity for VOCs compared with other sites may be dependent on the amount of acylcarnitines incorporated into the sarcolemma compared with other organelles, and on the experimental conditions.

This effect will therefore have considerable clinical import for the pathogenesis of ischaemia. For example, in ischaemia VOCs activated by acyl carnitines would be relatively resistant to compounds such as verapamil, diltiazem and nifedipine, although not to class III calcium-antagonists (calcium-overload blockers). Acyl carnitines may therefore be responsible for some of the massive Ca²⁺ overload which follows reperfusion of ischaemic tissues. Indeed, infusion of palmitoyl carnitine (50 µmol l⁻¹) into the coronary artery of cats produces ST segment elevation, arrhythmias, ventricular fibrillation and death; the effects were similar to, and had the same time course as, coronary ligation (Bentham *et al.*, 1986). Lysophosphatidyl choline was also infused during these experiments and although similar arrhythmias occurred, there was not the same extent of ST segment elevation, perhaps due to less myocardial work.

In conclusion, palmitoyl carnitine, an endogenous occurring lipid metabolite, has been shown to resemble Bay K 8644 in a variety of interactions at VOCs, although the molecular site of action does not appear to be at the dihydropyridine binding site. Palmitoyl carnitine may be considered to be an endogenous activator of VOCs.

References

- ADAMS, R.J., COHEN, D.W., GUPTE, S., JOHNSON, J.D., WALLICK, E.T., WANG, T. & SCHWARTZ, A. (1979). In vitro effects of palmitylcarnitine on cardiac plasma membrane Na,K-ATPase, and sarcoplasmic reticulum Ca²⁺-ATPase and Ca²⁺ transport. *J. biol. Chem.*, **254**, 12404–12410.
- ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.
- BENTHAM, J.M., HIGGINS, A.J. & WOODWARD, B. (1986). Effects of lipid amytriphiles on arrhythmogenesis in the anaesthetized cat. *Br. J. Pharmac.*, **89**, 735P.
- BIGAUD, M. & SPEDDING, M. (1986). Inhibition of the effects of endothelial-derived relaxant factor (EDRF) in aorta by palmitoyl carnitine. *Br. J. Pharmac.*, **89**, 540P.
- BODDEKE, H.W.G.M., HEYNIS, J.B., WILFFERT, B. & VAN ZWIETEN P.A. (1987). A study on the mechanism of negative inotropy of some calcium entry blockers in isolated guinea-pig hearts. *Br. J. Pharmac.*, **90**, 131P.
- BRECHER, P. (1983). The interaction of long-chain acyl CoA with membranes. *Mol. Cell. Biochem.*, **57**, 3–15.
- BRUM, G., FLOCKERZI, V., HOFMANN, F., OSTERRIEDER, W. & TRAUTWEIN, W. (1983). Injection of catalytic subunit of cAMP-dependent protein kinase into isolated cardiac myocytes. *Pflugers Arch.*, **398**, 147–157.
- CORR, P.B., SNYDER, D.W., CAIN, M.E., CRAFFORD, JR, W.A., GROSS, R.W. & SOBEL, B.E. (1981). Electrophysiological effects of amphiphiles on canine Purkinje fibers. Implications for dysrhythmia secondary to ischaemia. *Circulation Res.*, **49**, 354–363.
- CORR, P.B., SNYDER, D.W., LEE, B.I., GROSS, R.W., KEIM, C.R. & SOBEL, B.E. (1982). Pathophysiological concentrations of lysophosphatides and the slow response. *Am. J. Physiol.*, **243**, H187–H195.
- CORR, P.B., GROSS, R.W. & SOBEL, B.E. (1984). Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circulation Res.*, **55**, 135–154.
- DUNCAN, G.P., PATMORE, L. & SPEDDING, M. (1986). Positive inotropic effects of palmitoyl carnitine on embryonic chick heart cell aggregates. *Br. J. Pharmac.*

- 89, 757P.
- ESKELINEN, S. & MELA, M. (1984). Cation permeability and mechanical properties of the erythrocyte membrane under the influence of lysophosphatidylcholine (LPC) in isotonic and hypotonic media. *Acta Physiol. Scand.*, **122**, 527–534.
- GALIZZI, J.-P., FOSSET, M. & LAZDUNSKI, M. (1985). Characterization of the Ca²⁺ coordination site regulating binding of Ca²⁺ channel inhibitors d-cis-diltiazem, (+)bepridil and (–)desmethoxyverapamil to their site in skeletal muscle transverse tubule membranes. *Biochem. biophys. Res. Comm.*, **132**, 49–55.
- GLOSSMANN, H., FERRY, D.R., GOLL, A., STRIESNIG, J. & ZERNIG, G. (1985). Calcium channels and calcium channel drugs: recent biochemical and biophysical findings. *Arzneim-Forsch.*, **35**, 1917–1935.
- GODFRAIND, T. & MILLER, R.C. (1983). Specificity of action of Ca²⁺ entry blockers. A comparison of their actions in rat arteries and in human coronary arteries. *Circulation Res.*, **52**, suppl I, 81–91.
- GRIMA, M., SCHWARTZ, J., SPACH, M.O. & VELLY, J. (1986). Antianginal arylalkylamines and sodium channels: [³H]-batrachotoxin-A 20-alpha-benzoate and [³H]-tetracaine binding. *Br. J. Pharmac.*, **89**, 641–646.
- HARRIS, K.M., KONGSAMUT, S. & MILLER, R.J. (1986). Protein kinase C mediated regulation of calcium channels in PC-12 pheochromocytoma cells. *Biochem. biophys. Res. Comm.*, **134**, 1298–1305.
- HELFMAN, D.M., BARNES, K.C., KINKADE, Jr. J.M., VOGLER, W.R., SHOJI, M. & KUO, J.F. (1983). Phospholipid-sensitive Ca²⁺-dependent protein phosphorylation system in various types of leukemic cells from human patients and in human leukemic cell lines HL60 and K562, and its inhibition by alkyl-lysophospholipid. *Cancer Res.*, **43**, 2955–2961.
- HILLE, B. (1984). *Ionic Channels of Excitable Membranes*. Sunderland, Mass: Sinauer.
- HOLZ IV, G.G., RANE, S.G. & DUNLAP, K. (1986). GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature*, **319**, 670–672.
- IDELL-WENGER, J.A., GROTYOHANN, L.W. & NEELY, J.R. (1978). Coenzyme A and carnitine distribution in normal and ischemic hearts. *J. Biol. Chem.*, **253**, 4310–4318.
- INOUE, D. & PAPPANO, A.J. (1983). L-Palmitoylcarnitine and calcium ions act similarly on excitatory ionic currents in avian ventricular muscle. *Circulation Res.*, **52**, 625–634.
- JANIS, R.A. & TRIGGLE, D.J. (1984). 1,4-Dihydropyridine Ca²⁺ channel antagonists and activators: a comparison of binding characteristics with pharmacology. *Drug Dev. Res.*, **4**, 257–274.
- KATOH, N., WRENN, R.W., WISE, B.C., SHOJI, M. & KUO, J.F. (1981). Substrate proteins for calmodulin sensitive Ca²⁺-dependent protein kinases in heart, and inhibition of their phosphorylation by palmitoylcarnitine. *Proc. natn. Acad. Sci., U.S.A.*, **78**, 4813–4817.
- KATZ, A.M. & MESSINEO, F.C. (1981). Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. *Circulation Res.*, **48**, 1–16.
- KNABB, M.T., SAFFITZ, J.E., CORR, P.B. & SOBEL, B.E. (1986). The dependence of electrophysiological derangements on accumulation of endogenous long-chain acyl carnitine in hypoxic neonatal rat myocytes. *Circulation Res.*, **58**, 230–240.
- KURIEN, V.A. & OLIVER, M.F. (1966). Serum-free-fatty-acids after acute myocardial infarction and cerebral vascular occlusion. *Lancet*, **ii**, 122–127.
- DELEIRIS, J. & FEUVRAY, D. (1977). Ischaemia-induced damage in the working rat heart preparation: the effect of perfusate substrate composition upon subendocardial ultrastructure of the ischaemic left ventricular wall. *J. Mol. Cell Cardiol.*, **9**, 365–373.
- LIEDTKE, A.J., NELLIS, S. & NEELY, J.R. (1978). Effects of excess free fatty acids on mechanical and metabolic function in normal and ischemic myocardium in swine. *Circulation Res.*, **43**, 652–661.
- LINDEN, J. & BROOKER, G. (1980). The influence of resting membrane potential on the effect of verapamil on atria. *J. Mol. Cell Cardiol.*, **12**, 325–331.
- MANNHOLD, R., RODENKIRCHEN, R., BAYER, R. & HAAS, W. (1984). The importance of drug ionization for the action of calcium-antagonists and related compounds. *Arzneim-Forsch.*, **34**, 407–409.
- MESSING, R.O., CARPENTER, C.L. & GREENBERG, D.A. (1986). Inhibition of calcium flux and calcium channel antagonist binding in the PC 12 neural cell line by phorbol esters and protein kinase C. *Biochem. biophys. Res. Comm.*, **136**, 1049–1056.
- MIR, A.K. & SPEDDING, M. (1986). Palmitoyl carnitine, a lipid metabolite produced in ischaemia, activates Ca²⁺ channels in smooth muscle. *Br. J. Pharmac.*, **88**, 381P.
- NEELY, J.R. & FEUVRAY, D. (1981). Metabolic products and myocardial ischemia. *Am. J. Pathol.*, **102**, 282–291.
- OSUGI, T., IMAIZUMI, T., MIZUSHIMA, A., UCHIDA, S. & YOSHIDA, H. (1986). 1-Oleoyl-2-acetyl-glycerol and phorbol diester stimulate Ca²⁺ influx through Ca²⁺ channels in neuroblastoma x glioma hybrid NG 108–15 cells. *Eur. J. Pharmac.*, **126**, 47–51.
- PALFREYMAN, M.G., MIR, A.K., KUBINA, M., MIDDLEMISS, D.N., RICHARDS, M., TRICKLEBANK, M.D. & FOZARD, J.R. (1986). Monoamine receptor sensitivity changes following chronic administration of MDL 72394, a site-directed inhibitor of monoamine oxidase. *Eur. J. Pharmac.*, **130**, 73–89.
- PHILIPSON, K.D. (1984). Interaction of charged amphiphiles with Na⁺-Ca²⁺ exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.*, **259**, 13999–14002.
- PHILIPSON, K.D., LANGER, G.A. & RICH, T.L. (1985). Charged amphiphiles regulate heart contractility and sarcolemma-Ca²⁺ interactions. *Am. J. Physiol.*, **248**, H147–H150.
- PIPER, M.H., SEZER, O., SCHWARTZ, P., HUTTER, J.F., SCHWEICKHARDT, C. & SPIECKERMANN, P.G. (1984). Acyl-carnitine effects on isolated cardiac mitochondria and erythrocytes. *Basic Res. Cardiol.*, **79**, 186–198.
- PITTS, B.J.R., TATE, C.A., VAN WINKLE, W.B., WOOD, J.M. & ENTMAN, M.L. (1978). Palmitoylcarnitine inhibition of the calcium pump in cardiac sarcoplasmic reticulum: a possible role in myocardial ischemia. *Life Sci.*, **23**, 391–402.
- REUTER, H. (1967). The dependence of slow inward current in Purkinje fibres on the extracellular calcium concentration. *J. Physiol.*, **192**, 479–492.
- REYNOLDS, I.J., GOULD, R.J. & SNYDER, S.H. (1983). [³H]-Verapamil binding sites in brain and skeletal muscle: regulation by calcium. *Eur. J. Pharmac.*, **95**, 315–321.
- SCHRAMM, M. & TOWART, R. (1984). Modulation of calcium channel function with 1,4-dihydropyridine structure. *J. Physiol.*, **349**, 53P.

- SCHRAMM, M., THOMAS, G., TOWART, R. & FRANCK-OWIAK, G. (1983). Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature*, **309**, 535–537.
- SPEDDING, M. (1982). Assessment of 'Ca²⁺-antagonist' effects of drugs in K⁺-depolarized smooth muscle. Differentiation of antagonist subgroups. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **318**, 234–240.
- SPEDDING, M. (1984). Changing surface charge with salicylate differentiates between subgroups of calcium-antagonists. *Br. J. Pharmac.*, **83**, 211–220.
- SPEDDING, M. (1985a). Competitive interactions between Bay K 8644 and nifedipine in K⁺ depolarized smooth muscle: a passive role for Ca²⁺? *Naunyn-Schmiedeberg's Arch. Pharmac.*, **328**, 464–466.
- SPEDDING, M. (1985b). Activators and inactivators of Ca²⁺ channels: new perspectives. *J. Pharmac. (Paris)*, **16**, 319–343.
- SPEDDING, M. & BERG, C. (1984). Interactions between a 'calcium channel agonist' Bay K 8644, and calcium antagonists differentiate calcium antagonist subgroups in K⁺-depolarized smooth muscle. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **328**, 69–75.
- SPEDDING, M., SCHINI, V., SCHOEFFTER, P. & MILLER, R.C. (1987). Calcium channel activation does not increase the release of endothelial-derived relaxant factor (EDRF) although tonic release of EDRF may modulate calcium channel activity in smooth muscle. *J. cardiovasc. Pharmac.*, **8**, 1130–1137.
- SPERALAKIS, N. & SCHNEIDER, J.A. (1976). A metabolic control mechanism for calcium ion influx that may protect the ventricular myocardial cell. *Am. J. Cardiol.*, **37**, 1079–1085.
- SU, C.M., SWAMY, V.C. & TRIGGLE, D.J. (1984). Calcium channel activation in vascular smooth muscle by Bay K 8644. *Can. J. Physiol. Pharmac.*, **62**, 1401–1410.
- THOMAS, G., GROSS, R. & SCHRAMM, M. (1984). Calcium channel modulation: ability to inhibit or promote calcium influx resides in the same dihydropyridine molecule. *J. cardiovasc. Pharmac.*, **6**, 1170–1176.
- TSIEN, R.W., GILES, W. & GREENGARD, P. (1972). Cyclic AMP mediates the effects of adrenaline on cardiac Purkinje fibres. *Nature, New Biol.*, **240**, 181–183.
- WAKADE, A.R., MÁLHROTA, R.K. & WAKADE, T.D. (1986). Phorbol ester facilitates ⁴⁵Ca accumulation and catecholamine secretion by nicotine and excess K⁺ but not by muscarine in rat adrenal medulla. *Nature*, **321**, 698–700.
- WINER, B.J. (1962). *Statistical Principles in Experimental Design*. New York: McGraw Hill.
- WISE, B.C., GLASS, D.B., CHOU, C-H.J., RAYNOR, R.L., KATOH, N., SCHATZMAN, R.C., TURNER, R.S., KIBLER, R.F. & KUO, J.F. (1982). Phospholipid-sensitive Ca²⁺-dependent protein kinase from heart. II Substrate specificity and inhibition by various agents. *J. biol. Chem.*, **257**, 8489–8495.
- WISE, B.C. & KUO, J.F. (1983). Modes of inhibition by acylcarnitines, adriamycin and trifluoperazine of cardiac phospholipid-sensitive calcium-dependent protein kinase. *Biochem. Pharmac.*, **32**, 1259–1265.

(Received April 29, 1987.

Revised June 13, 1987.

Accepted June 19, 1987.)