Br. J. Pharmacol. (1988), **95**, 771–776

Effects of calcium channel antagonists and facilitators on beating of primary cultures of embryonic chick heart cells

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1 Primary aggregate cultures of embryonic chick heart have been used to investigate the effects of calcium channel antagonists and facilitators on myocardial contractility.

2 The number of aggregates showing movement was inhibited in a concentration-dependent manner by calcium antagonists from different subgroups with negative log concentrations inhibiting movement in 50% of aggregates as follows: Class 1 – nisoldipine (7.20); Class 2 – verapamil (6.36), diltiazem (5.83); Class 3 – lidoflazine (5.68), pimozide (6.25).

3 The effects of the dihydropyridine facilitators Bay K 8644 and CGP 28392 on aggregate beating were investigated by evaluating the interaction between calcium channel facilitators and antagonists from the three subgroups of calcium antagonists. Concentrations of antagonists that inhibited beating in 85% of aggregates were used. Both Bay K 8644 and CGP 28392 reversed nisoldipine-, diltiazem- or verapamil-induced inhibition of beating.

4 Bay K 8644 was approximately 10 times more potent than CGP 28392 in reversing nisoldipine-, diltiazem- or verapamil-induced inhibition of beating.

5 For each facilitator the concentrations causing 50% reversal of inhibition of aggregate beating against the three antagonists were similar. There was little evidence for differential modulation by verapamil or diltiazem of the action of the dihydropyridine facilitators.

6 Bay K 8644 did not reverse lidoflazine- or pimozide-induced inhibition of beating, indicating that these drugs may act at a site distinct from the dihydropyridine site on the calcium channel.

Introduction

Specific binding sites for radiolabelled calcium antagonists have been identified in smooth muscle, cardiac muscle and neuronal tissue (Triggle & Janis, 1984). In cardiac muscle, specific dihydropyridine binding sites have been characterized in whole cell binding studies in chick heart (Renaud *et al.*, 1984) and in embryonic chick cultured heart cells (Marsh *et al.*, 1983). These authors have correlated the binding of antagonists with the negative inotropic effects of these compounds, thus illustrating a functional site of action of antagonists at the calcium channel. Furthermore, Marsh *et al.* (1983) have suggested that it is the modulation of the low affinity dihydropyridine site which correlates with the negative inotropic activity of nitrendipine. In embryonic rat myocytes, Bellemann (1984) has also demonstrated competition between the dihydropyridine antagonist nimodipine and 'agonist' Bay K 8644 for the same low affinity specific receptor site associated with the calcium channel ([^{3}H]-nitrendipine displacement by Bay K 8644, K_a = 35 nM). Allosteric modulation of dihydropyridine binding by diltiazem and verapamil has been reported by Yamamura *et al.* (1982) in synaptosomes and by Ehlert *et al.* (1982) in rat brain and heart membranes. In contrast Marsh *et al.* (1983) found no evidence for interaction between diltiazem and the dihydropyridine binding site on intact chick heart cells. Similarly, Spedding & Berg (1984) found that verapamil or diltiazem caused minimal allosteric modulation of the action of the dihydropyridine Bay K 8644 on contractions of taenia caeci smooth muscle. Spedding & Berg (1984) did, however, find interesting differences between the action of the calcium channel facilitator Bay K 8644 and different subgroups of calcium antagonists. These authors found competitive interactions with dihydropyridine antagonists (class 1, Spedding, 1985), non-competitive interactions with the antagonists diltiazem and verapamil (class 2) and a lack of interaction with diphenylalkylamine antagonists

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(class 3). The data indicated that the subgroups of calcium antagonists have distinct sites of action on the smooth muscle calcium channel. In our present work we have quantified the inhibitory effects of representative antagonists from each of the three subgroups on the beating of embryonic chick heart cell aggregate cultures. Furthermore, we have investigated the reversibility of these antagonists by two dihydropyridine calcium channel facilitators, Bay K 8644 and CGP 28392. Our aim was to establish whether the action of these molecules is allosterically modulated by verapamil or diltiazem and whether the diphenylalkylamine antagonists have distinct sites of action on calcium channels in heart muscle. Preliminary accounts of this work have been communicated to the British Pharmacological Society (Clarke *et al.*, 1984; Duncan & Patmore, 1986; Duncan *et al.*, 1987).

Methods

Cell cultures

Eleven day old Leghorn K chicken embryos were killed by decapitation and the hearts removed under aseptic conditions. Ventricles were chopped into fragments and were washed in Hanks calcium- and magnesium-free balanced salt solution (Gibco) and then agitated at 37°C in the presence of 0.05% trypsin as described by Clusin (1981). Trypsinisation was then repeated 3–4 times and the dispersed cells were pooled, centrifuged and resuspended in culture media containing 20% nutrient media M-199 (Gibco), 6% heat-inactivated foetal calf serum, 1% penicillin/streptomycin solution and 73% low potassium salt solution the composition of which was (mM): NaCl 116, $NaHCO_3$ 26.2, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $CaCl_2$ 1.8 and dextrose 5.5 adjusted to pH 7.3. The cells were seeded at a density of 3–4 $\times 10^5$ cells ml^{-1} into 96 well trays. The bottom of each well was coated with a layer of Sylgard 184 (Dow Corning) silicon elastomer resin. This process inhibited the formation of monolayers and over a period of 3 days in culture the cells reaggregated to form roughly spherical clusters of 100–200 μm diameter (Figure 1).

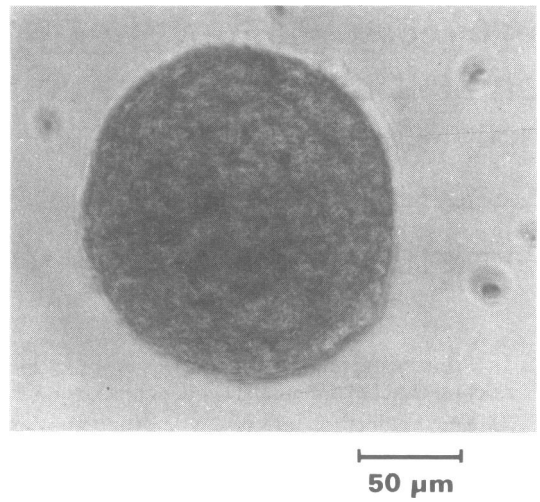

Figure 1 An aggregate of embryonic chick heart cells. Phase contrast photomicrograph of an aggregate of approximately 1000 cells. Sizes range from 100–200 μm diameter. These aggregates display automaticity with rates between 70 and 115 beats min^{-1} (mean = 107 ± 4, $n = 20$) which can be observed clearly under 40 × magnification.

Assessment of contractility and experimental procedures

Aggregates of cardiac cells displayed automaticity and the edge movement associated with contractions could be detected in each cluster on observation under low power (40 ×) microscopy. Approximately 15 aggregates formed in each culture well and contractility was assessed by scoring the % of aggregates in each well which were beating. This was done on an all-or-nothing basis and 10 randomly identified aggregates were scored. Control beating was normally 100%. Drugs were applied at 11 times the final concentration in a volume of 20 μl of salt solution, to wells containing 200 μl of media. The salt solution was designed to resemble the ionic composition of the culture medium and contains (mM): NaCl 110, KCl 1.07, $CaCl_2$ 1.7, $MgSO_4$ 0.73, HEPES 19.3, $NaHCO_3$ 0.83 and dextrose 5.5. Following drug application, cultures were incubated for 30 min at 37°C and then identified wells scored again for % of aggregates beating. Different treatments (drugs or concentrations) were applied to each of 40 wells. Replicate tests for each concentration of each drug were made on 3 further trays. On each tray a series of wells were treated as 'sham' controls where salt solution containing no drug was added. This normally caused no inhibition of beating. Inhibition-concentration curves were calculated for drugs by assessing the % of aggregates in which beating was completely inhibited. This was expressed as a mean from the 4 replicate culture trays tested. Thus 40 aggregates were sampled for each data point. These values were treated as $n = 1$ and negative logarithms of concentrations causing inhibition of beating in 50% of aggregates (pIC_{50} values) were calculated from an iterative least squares fit of a sigmoidal function to these data. Data shown are mean pIC_{50}

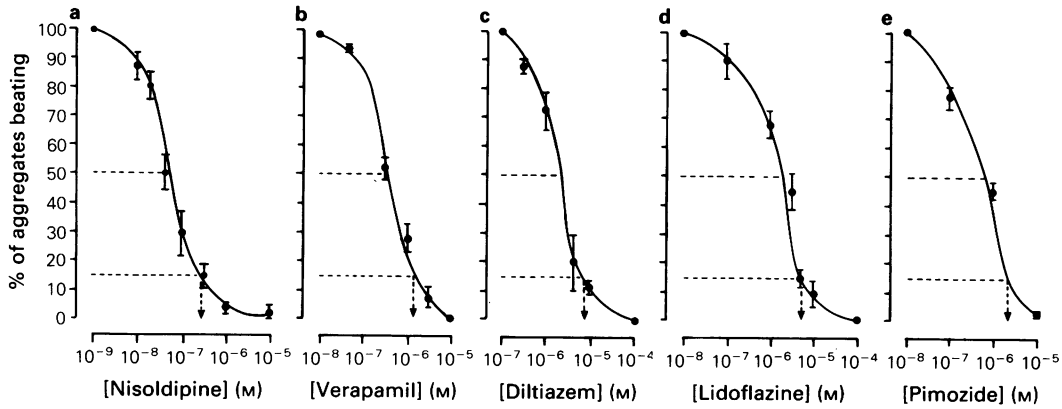

Figure 2 Inhibition-concentration curves for calcium antagonists. Data shown are mean values with s.e. of mean shown by vertical bars for (a) nisoldipine ($n = 4$), (b) verapamil ($n = 4$), (c) diltiazem ($n = 4$), (d) lidoflazine ($n = 6$) and (e) pimozide ($n = 4$). Sigmoidal curves were fitted to the raw data. pIC_{50} and IC_{85} values were calculated for each drug (see Tables 1 and 2).

values from such experiments performed on at least 4 separate cultures prepared from different egg batches. In experiments where competition between calcium channel facilitators and antagonists was investigated, concentrations of antagonists which caused approximately 85% inhibition of beating were added simultaneously with various concentrations of calcium channel facilitator. Competition between the facilitators and representative antagonists from the three subgroups of calcium antagonists (Spedding, 1985) were investigated. Class 1, nisoldipine (0.3 μM); class 2, verapamil (1 μM), diltiazem (10 μM); class 3, lidoflazine (7 μM), pimozide (3 μM). The scoring protocol was the same as that described for the previous experiments and the data were again expressed as % of the sham-treated control aggregate beating. Data were fitted to a sigmoidal function between 15 and 100% of control and negative logarithms of concentrations causing 50% (actually taken at 57.5% of control) reversal of antagonist-induced inhibition of aggregate beating (pEC_{50} values) calculated to express the potency of the calcium facilitators reversal of antagonist-induced inhibition of beating.

Table 1 Calcium antagonist potencies for inhibition of beating of embryonic chick heart cell aggregates

Compound	(n)	*pIC_{50}* (M)	(*s.e.range*)
Nisoldipine	4	7.20	(7.13–7.25)
Verapamil	30	6.36	(6.40–6.32)
Diltiazem	25	5.83	(5.86–5.80)
Lidoflazine	6	5.68	(5.87–5.68)
Pimozide	4	6.25	(6.31–6.21)

Drugs

The following drugs were used: nisoldipine, Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer), verapamil, diltiazem (Sigma), lidoflazine and

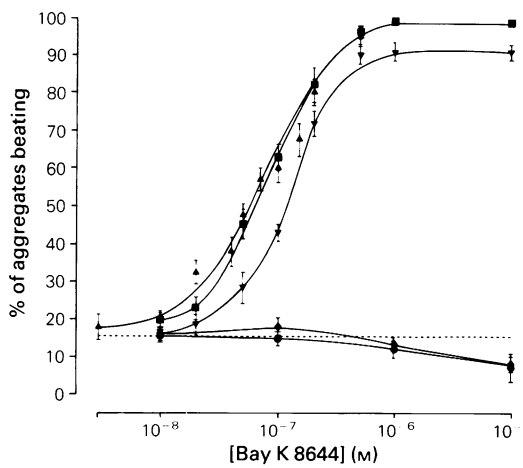

Figure 3 Reversal of antagonist-induced inhibition of beating by the calcium facilitator Bay K 8644. Aggregates were incubated with Bay K 8644 and concentrations of antagonists that inhibited beating in 85% of aggregates were calculated: nisoldipine (▲, 0.3 μM); verapamil (■, 1 μM); diltiazem (▼, 10 μM); lidoflazine (●, 7 μM) and pimozide (◆, 3 μM). Values shown are means from 4 separate experiments with s.e.mean shown by vertical bars. The broken line represents the average control level of inhibition of aggregate beating by the antagonists (no reversal by Bay K 8644).

pimozide (Jansen) and CGP 28392 (ethyl 4-[2-(difluoro - methoxy)phenyl] - 1,4,5,7 - tetrahydro - 2 - methyl - 5 - oxofuro[3,4 - b]pyridine - 3 - carboxylate Ciba Geigy). Dihydropyridines were dissolved in ethanol and diluted (at least 1:1000) with the salt solution. All other drugs were dissolved in distilled water and then added to the salt solution.

Results

The results show that calcium antagonists from all 3 subgroups inhibited aggregate beating in a concentration-dependent manner. Concentration-response curves for nisoldipine, verapamil, diltiazem, lidoflazine and pimozide are shown in Figure 2. The steep nature of the curves is due to the all-or-none endpoint of the measurement technique. From the curves pIC_{50} values were calculated. These values are shown in Table 1. The effects of the calcium channel facilitators Bay K 8644 (Schramm *et al.*, 1983a,b) and CGP 28392 (Erne *et al.*, 1984; Rogg *et al.*, 1985) were examined by evaluating the interaction between antagonists and facilitators.

The reversal of nisoldipine-induced inhibition of beating by Bay K 8644 is shown in Figure 3. Relative potencies for Bay K 8644 and CGP 28392 against these antagonists are shown in Table 2. Both Bay K 8644 (0.01–10 μM) and CGP 28392 (0.1–100 μM) reversed nisoldipine-, verapamil- and diltiazem-induced inhibition of beating. Potency values (pEC_{50}s) for Bay K 8644 were similar for the three antagonists (around 0.1 μM) with similar slopes of the concentration-response curves. The facilitator CGP 28392 was approximately 10 times less potent than Bay K 8644 in competing with antagonist-induced inhibition of aggregate beating. For all three antagonists, EC_{50} values were around 1 μM. A statistical analysis of the data by an unpaired Student's *t* test revealed no significant difference between EC_{50} values calculated from interactions between either Bay K 8644 or CGP 28392 and nisoldipine, verapamil or diltiazem. In contrast to the interaction or reversal observed between the facilitators and the class 1 and 2 calcium antagonists, Bay K 8644 did not reverse the inhibitory effects of the class 3 antagonists pimozide and lidoflazine.

Discussion

It has previously been shown that the contractility of embryonic chick heart cell aggregates is sensitive to calcium entry and can be inhibited by calcium antagonists (Marsh *et al.*, 1983; Patmore & Whiting, 1985; Patmore, 1986). These workers made direct measurements of contractility. However, the measurement technique which we have used in these experiments does not determine negative inotropic effects of calcium antagonists but defines concentrations that totally inhibit beating. Calcium antagonists exert a negative inotropic rather than negative chronotropic effects on embryonic chick myocytes. This can be seen in the time-dependent inhibition of contractility caused by verapamil (Barry & Smith, 1982; Patmore & Whiting, 1985) and by nitrendipine (Marsh *et al.*, 1983). Since individual aggregates have varying sensitivities, graded inhibition-concentration curves have been established. We have previously shown (Clarke *et al.*, 1984) that pIC_{50} values obtained by this method correlate well with pIC_{50} values determined from measurements of the negative inotropic effects of calcium antagonists on guinea-pig papillary muscle. However, cell culture estimates of pIC_{50} are lower than those obtained from papillary muscle (slope of regression line = 1.7). This might be expected due to the all-or-none end point of the former assay compared with the graded inhibition of contraction measured from ventricular fibres.

The dihydropyridines Bay K 8644 and CGP 28392 have been shown by a number of authors to facilitate calcium entry into muscle cells and Bay K 8644 has been shown to increase the magnitude of the slow inward current in cardiac muscle by facilitating calcium influx by increasing channel open

Table 2 Calcium facilitator potencies for reversal of antagonist-induced inhibition of beating

		Facilitators					
		Bay K 8644			*CGP 28392*		
Antagonists	IC_{85} (μM)	pEC_{50}	n	(*s.e.range*)	pEC_{50}	n	(*s.e.range*)
Nisoldipine	0.3	7.14	4	(7.11–7.16)	6.16	4	(6.06–6.28)
Verapamil	1.0	7.06	4	(7.01–7.10)	6.15	4	(6.04–6.29)
Diltiazem	10.0	6.89	4	(6.78–6.99)	5.71	4	(5.59–5.88)
Pimozide	3.0	NC	4				ND
Lidoflazine	7.0	NC	4				ND

NC = not calculable: no reversal of antagonist-induced inhibition of beating was observed. ND = not determined. *n* values shown represent data from *n* separate cultures prepared from different egg batches. For each culture, experiments were performed in quadruplicate.

state probability and open time (Hess *et al.*, 1984; Brown *et al.*, 1984; Nowycky *et al.*, 1985). In embryonic chick heart cell aggregates this results in an increase in contractility by Bay K 8644 when direct measurements of edge movement are made (Patmore & Whiting, 1986). However, without this sophisticated technique, visual observation of aggregates treated with Bay K 8644 yields only a qualitative impression of increased contractility. We have thus investigated the properties of Bay K 8644 and CGP 28392 by studying their competition with various antagonists. Specific binding sites for ^{3}H-labelled 1,4-dihydropyridines have been previously identified in cardiac muscle (Triggle & Janis, 1984) and thus these competition experiments may be compared with binding studies where the end point is a functional measurement of contractility rather than of bound ligand. Both Bay K 8644 and CGP 28392 reversed the inhibition of beating caused by the dihydropyridine antagonist nisoldipine suggesting displacement of nisoldipine from its site on the calcium channel. The affinity of these sites on cerebral cortex membranes has been shown to be allosterically modulated, negatively by verapamil and positively by diltiazem. However, pEC_{50} values obtained with Bay K 8644 or CGP 28392 against either nisoldipine, verapamil or diltiazem were not significantly different. Thus there is little evidence for differential allosteric modulation by verapamil or diltiazem of the binding site of these dihydropyridine facilitators in these functional experiments on heart muscle. These findings agree with the observations of Spedding & Berg (1984) who found an absence of allosteric modulation of the action of Bay K 8644 in functional studies on taenia caeci smooth muscle. In the same preparations Spedding (1985) has shown competitive reversal of the effects of dihydropyridines with Bay K 8644 and non-competitive reversal of the inhibitory effects of verapamil and diltiazem. The present study provides no evidence to suggest whether the interaction between Bay K 8644 or CGP 28392 and these antagonists was competitive or non-competitive.

Spedding & Berg (1984) found that the effects of the diphenylalkylamine (class 3) antagonists was not reversed by Bay K 8644. We have found a similar lack of competition between Bay K 8644 and class 3 calcium antagonists. Bay K 8644 did not reverse the inhibitory effects of pimozide or lidoflazine. These observations may be explained by irreversible binding of the class 3 calcium antagonists to the dihydropyridine site on the calcium channel. We consider that this is unlikely since in smooth muscle Spedding & Berg (1984) found no difference in apparent pA_2 values for the class 3 calcium antagonist cinnarizine obtained in the presence or absence of Bay K 8644 suggesting that the class 3 antagonists do not behave in an irreversible manner. As the site for the class 3 calcium antagonists appears not to be the same as that for the dihydropyridines, then either the drugs must act at a site that is not on the calcium channel or alternatively interact with a site or state of the channel that is inaccessible to calcium channel facilitators.

We would like to thank Bayer for the gifts of nisoldipine and Bay K 8644, Ciba Geigy for CGP 28392 and Jansen for lidoflazine and pimozide.

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(Received March 25, 1988
Revised May 26, 1988
Accepted June 10, 1988)