RS 30026: a potent and effective calcium channel agonist

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- 1 A series of dihydropyridine derivatives has been evaluated for calcium channel agonist activity using reversal of nisoldipine-induced inhibition of beating of aggregates of embryonic chick myocytes. This test appears to be specific for calcium channel agonists since isoprenaline and cardiac glycosides are inactive.
- 2 RS 30026 was the most potent of the series, was significantly more potent than CGP 28392 and of similar potency to Bay K 8644 (pEC_{50} = 7.45, 6.16 and 7.20, respectively). RS 30026 increased edge movement of individual aggregates, in the absence of nisoldipine, by 50% at 2 nM.
- 3 Compounds were also evaluated for their effects on guinea-pig papillary muscle and porcine coronary artery rings. RS 30026 displayed positive inotropism at concentrations between 10^{-9} and 10^{-6} M (pEC_{200} = 8.21), but was a much more powerful inotrope than Bay K 8644, increasing contractility to 1300% of control at 10^{-6} M (compared to 350% of control for Bay K 8644). RS 30026 caused vasoconstriction at concentrations between 10^{-10} and 10^{-7} M.
- 4 Calcium channel currents in single embryonic chick myocytes were recorded by whole-cell voltage clamp techniques. RS 30026 (100 nM–500 nM) produced large increases in peak current amplitude and shifted the voltage for threshold and maximal currents to more negative values. RS 30026 (500 nM) also produced large increases in the inward tail currents evoked upon repolarization. The effects of Bay K 8644 (50 and 500 nM) were much less marked.
- 5 Analysis of the activation characteristics of currents showed parallel shifts in the activation curve to more negative potentials in the presence of 50 nM Bay K 8644, with a much smaller shift in the presence of 500 nM Bay K 8644. RS 30026 (100 and 500 nM) caused concentration-dependent shifts in the activation of the calcium channel currents with an increase of the slope of the curve.
- 6 RS 30026 appears to be the most potent and effective calcium channel agonist described to date.

Introduction

Schramm *et al.* (1983a) described the dihydropyridine Bay K 8644 which acted in the opposite manner to calcium channel antagonists, such as nifedipine and verapamil, in that it was positively inotropic and caused vasoconstriction. This compound and the dihydropyridine-lactone CGP 28392 have been shown to act directly at the calcium channel to increase the inward calcium current by increasing the probability of channel opening and the mean open time (Brown *et al.*, 1984; Hess *et al.*, 1984; Kokubun & Reuter, 1984; Nowicky *et al.*, 1985; Bechem & Schramm, 1987). In all studies CGP 28392 appears to be less potent and effective than Bay K 8644 (Erne *et al.*, 1984; Renaud *et al.*, 1984; Rogg *et al.*, 1985; Patmore & Duncan, 1988). In heart and neurones Bay K 8644 has been shown to shift the activation curve for the transient inward current to the left and increase the magnitude of the peak current (Sanguinetti *et al.*, 1986; Docherty & Brown, 1986; Hamilton *et al.*, 1987). However, large increases in calcium influx as a consequence of the increased open time of the channel result in larger tail currents being observed at the end of voltage clamp pulses. Under physiological rather than voltage clamp conditions the contributions from different types of calcium current will depend on the spike or action potential characteristics (Anderson & Patmore, 1988) and on the type of tissue and its resting membrane potential. Thus in neurones, which have short action potentials, the calcium current will be less affected than in smooth muscle, where many cells display slow spike activity. The more positive resting potential in smooth muscle is also closer to the thresh-

old activation potential for the calcium current, which is shifted to more negative potentials in the presence of Bay K 8644.

The actions of dihydropyridine calcium channel agonists mirror the activity of the antagonists in that they display an apparent selectivity for vascular smooth muscle over cardiac muscle (Clarke & Patmore, 1986; Kwon *et al.*, 1989) and selectively enhance resistance artery myogenic tone (Hwa & Bevan, 1986). As potential inotropes these compounds are likely to cause vasoconstriction at lower concentrations than they would be positively inotropic.

It has been shown that low concentrations of dihydropyridine calcium channel antagonists can display agonist effects (Brown *et al.*, 1986) and at high concentrations racemic Bay K 8644 behaves as an antagonist (Thomas *et al.*, 1984). This appears to be due, at least in part, to the fact that one of the stereo-isomers displays agonist activity. Thus the (+)-isomer of Bay K 8644 has been shown to behave as an antagonist whilst the (–)-isomer displayed agonist activity (Franckowiak *et al.*, 1985; Hamilton *et al.*, 1987; MacDonald *et al.*, 1987). Interestingly, the actions of the isomers are not so clear, since MacDonald *et al.* (1987) demonstrated that at high concentrations the (–) (agonist)-isomer of Bay K 8644 displayed antagonist activity. Similarly the (+)- and (–)-isomers of PN 202-791 have been described as agonist and antagonist respectively (Hof *et al.*, 1985; Kongsamut *et al.*, 1985; Williams *et al.*, 1985). In the present study we have evaluated the agonist activity of a series of dihydropyridine-lactone compounds related to CGP 28392. We have tested for agonist or facilitator activity by assessing the ability of compounds to reverse nisoldipine-induced inhibition of beating of aggregates of embryonic chick myocytes, as described by Patmore & Duncan (1988). The most potent compound of the series has been evaluated for its vasoconstrictor and positive inotropic

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activities and for its actions on the calcium channel currents in myocytes. A preliminary account of the effects of RS 30026 on cardiac calcium channel currents has been presented to the Physiological Society (Anderson & Patmore, 1990).

Methods

Cell cultures

Primary cultures of myocytes were prepared as described previously by Patmore & Duncan (1988). Briefly, myocytes were dispersed from the hearts of eleven day old Leghorn K chicken embryos by agitation in Hanks calcium- and magnesium-free balanced salt solution (Gibco) with 0.05% trypsin. Cells were 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, NaHCO₃ 26.2, MgSO₄ 0.8, NaH₂PO₄ 0.9, CaCl₂ 1.8 and dextrose 5.5, adjusted to pH 7.3. For measurements of antagonist-facilitator interactions and edge movement the cells were seeded into 96 well trays and the bottom of each well was coated with a layer of silicon elastomer resin to promote the formation of aggregates of 100–200 µm diameter. For electrophysiological experiments the cells were plated onto 35 mm plastic petri dishes at low density to allow recording from isolated myocytes. Cultures were maintained for 2–4 days before use.

Assessment of beating

Competition experiments between nisoldipine and potential calcium channel facilitators were performed as described by Patmore & Duncan (1988). Aggregates of myocytes displayed automaticity and the edge movement associated with contractions could be detected in each cluster on observation under low power (40×) microscopy. Contractility was assessed by scoring the % of aggregates in each well which were beating. 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 media and contained (mM): NaCl 110, KCl 1.07, CaCl₂ 1.7, MgSO₄ 0.73, HEPES 19.3, NaHCO₃ 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. For each concentration of each drug replicate tests 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 caused no inhibition of beating.

Competition between calcium channel facilitators and antagonists was investigated by treating aggregates with varying concentrations of facilitator and 0.3 µM nisoldipine, a concentration of antagonist which caused approximately 85% inhibition of beating. Calcium channel facilitators caused a concentration-dependent reversal of nisoldipine-induced inhibition of beating. Data were fitted to a sigmoidal function between 15 and 100% of control and negative logarithms of concentrations causing 50% reversal of antagonist-induced inhibition of aggregate beating (pEC₅₀ values) calculated to express the potency of the calcium channel facilitator. Where appropriate pEC₅₀ values were compared by a Student's *t* test.

Detection of edge movement

Contractility was estimated by measuring edge movement of aggregates of embryonic chick heart cells, as described by Patmore & Duncan (1988). Aggregates, initially grown on silicon elastomer, were transferred to normal tissue culture treated plates where they developed contacts with the dish

such that they could be superfused at 2.5 ml min⁻¹ (chamber volume = 200 µl) with a salt solution prewarmed to 37°C. The salt solution contained (mM): NaCl 110, KCl 1, MgSO₄ 0.8, HEPES 19.3, NaHCO₃ 0.83 and dextrose 5.5, pH adjusted to 7.4 with NaOH. An area of the aggregate which contained an edge which displayed significant movement was selected for analysis, by use of a video motion detection system described previously (Patmore & Whiting, 1985; Patmore *et al.*, 1989a). Dynamic recordings (sampled every 20 ms) of edge movement of the aggregate were recorded.

Electrophysiological recordings

Whole-cell voltage clamp recordings were made on single myocytes. Pipettes were made from Garner glass (Corning No. 7052) and filled with a solution containing (mM): CsCl 100, MgCl₂ 1, EGTA 11, CaCl₂ 1, HEPES 10 and glucose 5, pH 7.2. Experiments were carried out at room temperature. Recordings were made according to the methods of Hamill *et al.* (1981) by an EPC-7 amplifier (List Electronics, F.R.G.). Pipette resistances were 2–3 MΩ. No compensation for series resistance (R_s) was made. R_s was assessed to be at a maximum at 6 MΩ, based on the estimation of R_s during whole cell voltage clamp of twice the pipette resistance in the bath (Marty & Neher, 1983). Cells were superfused at a rate of 5–7 ml min⁻¹ with a solution containing (mM): NaCl 110, KCl 1, HEPES 20, MgCl₂ 0.8, glucose 5.5 and NaHCO₃ 10, pH 7.4 when gassed with O₂ containing 5% CO₂. BaCl₂ 10 mM was added to the pregassed solution to avoid precipitation. Tetrodotoxin (TTX, 0.5 µM; Sigma) was also added to the superfusate. Calcium channel currents were evoked every 15–20 s by rectangular depolarizations from a holding potential of -80 mV. Current records were stored on magnetic tape (Racal Store 4DS), filtered at 0.25 kHz and subsequently digitised through the 12-bit A-D converters of a CED 1401 interface linked to an IBM AT computer. For all records shown in this paper, linear leak and capacitive currents have been subtracted from the whole cell current record. Conductance data were fitted to a Boltzmann function by the Simplex method of Nelder & Mead (1964), as implemented in the sub-routine AMEOBA (Press *et al.*, 1986).

Guinea-pig papillary muscles

Female guinea-pigs (Dunkin Hartley, 200–300 g) were killed by cervical dislocation. Right ventricular papillary muscles were dissected from the hearts and placed in oxygenated ice-cold salt solution. The muscles were suspended in a chamber of volume 1.8 cm³ and superfused at 5 ml min⁻¹ with salt solution containing (mM): NaCl 145, KCl 6, CaCl₂ 2, MgCl₂ 1, Trizma buffer 10 and glucose 5.5, pH 7.4 at 37°C. The tendon end of the muscle was attached to a miniature force transducer (AME 875, Norway) and the preparation was stimulated at 1 Hz via platinum field electrodes (0.5 ms, 2× threshold voltage ~ typically 3 V). Muscles were stretched to generate maximum developed tension. Following a period of equilibration (1–1.5 h) the muscles were superfused with the drugs under test. Responses to increasing concentrations of a drug were displayed on a galvanometric chart recorder. Measurements of twitch magnitude were made after 30 min exposure to each drug concentration and were expressed as a percentage of the twitch magnitude at the end of the control (equilibration) period. From concentration-response curves, concentrations increasing contractility to 200% of control levels (EC₂₀₀) were calculated.

Porcine coronary artery rings

Porcine hearts were obtained from the local abattoir and left anterior descending coronary artery was dissected free. Rings of 2–3 mm length were cut and suspended between two fine needles, one of which was attached to a miniature force transducer (AME 875) in a perspex chamber (volume = 2 cm³) and

Figure 1 Molecular structures of Bay K 8644, CGP 28392 and RS 30026

superfused at 5 ml min^{-1} with salt solution (as described for papillary muscles) prewarmed to 37°C . The rings were placed under an initial resting tension of 1 g and were stimulated with 10 min pulses of high potassium (120 mM) salt solution at 30 min intervals. Increased potassium levels in these solutions were obtained by increasing the concentration of potassium at the expense of sodium. The preparations were allowed to equilibrate for 2–3 h, during which time successive high potassium responses of similar magnitude were obtained; this was taken as a maximal response. The rings were then superfused with 30 mM potassium salt solution to obtain a partial tonic contracture. These were typically around 30% of maximum; this was taken as a basal level for each tissue. Drugs were then added to the 30 mM potassium salt solution and increases in tonic contracture recorded. Measurements were made after 30 min drug exposure and increasing concentrations were applied to each tissue. Contractures were expressed as a percentage of the maximum contracture to 120 mM potassium observed during the equilibration period. Concentration-response curves were constructed and concentrations which increased the tone to 50% of the maximum level (EC_{50}) were calculated from an iterative fit of a sigmoid function to the data.

Drugs

Bay K 8644 (see Figure 1), RS 30026 (methyl 2-methyl-4-(2-benzoyloxyphenyl)-5-oxo-1,4,5,7-tetrahydrothieno [3,4-b] pyridine-3-carboxylate; Figure 1) and analogues were synthesised in the Institute of Organic Chemistry, Syntex Research, Palo Alto (Contribution No. 782). Details of the synthesis of RS 30026 will be published separately (R. Greenhouse). Both compounds were used as racemates. Nisoldipine was obtained from Bayer. All dihydropyridines were dissolved in ethanol and added to salt solutions to attain the concentrations required. The final concentration of ethanol was always less than 0.1%.

Results

Interactions between calcium channel antagonist and facilitators on cell beating

The use of embryonic chick heart cell aggregates in the investigation of interactions between calcium channel antagonists and facilitators has been previously described (Patmore & Duncan, 1988). We have examined the interactions between a novel series of dihydropyridines and the antagonist nisoldipine. From concentration-response relationships derived for each compound, a potency value has been calculated as the concentration causing reversal of inhibition of beating in 50% of the aggregates under test (pEC_{50}). Data for RS 30026 analogues and the standards Bay K 8644 and CGP 28392 are shown in Table 1. The RS 30026 series are based on the lactone-dihydropyridine structure of the calcium channel facilitator CGP 28392 (Figure 1). Reversal of nisoldipine-induced inhibition of beating by RS 30026 and Bay K 8644 is shown in Figure 2.

Table 1 Structure-activity relationships for RS 30026 series

RS No.	R ₁	R ₂	X	pEC ₅₀
Bay K 8644*				7.20
CGP 28392* [RS 71684]				6.16
31003	C ₂ H ₅	Cl	O	5.61
31227	C ₂ H ₅	Br	O	5.84
31617	C ₂ H ₅	CF ₃	O	6.03
71684	C ₂ H ₅	OCHF ₂	O	6.21
30421	C ₂ H ₅	Ph	O	5.83
31156	C ₂ H ₅	OCH ₂ Ph	O	6.67
30570	C ₂ H ₅	O(CH ₂) ₂ Ph	O	43% at 10^{-5} M
30321	C ₂ H ₅	O(CH ₂) ₃ Ph	O	5.95
31611	CH ₃	CH ₃	O	5.76
34674	CH ₃	OCH ₂ CH=CH ₂	O	5.18
34667	CH ₃	OPh	O	6.21
30124	CH ₃	OCH ₂ Ph	O	7.10
30026	CH ₃	OCH ₂ Ph	S	7.45
34389	CH ₃	Cl	S	6.15

Data shown are negative logarithms of concentrations of compounds causing a 50% reversal of nisoldipine-induced inhibition of beating. Values are calculated from a least squares non-linear regression fit to data from 4 experiments. Reproducibility of these potency values is shown by comparison of values for CGP 28392 *from Patmore & Duncan (1988) with that obtained for the same compound RS 71684 in a separate series of experiments. Only partial reversal (43%) of nisoldipine-induced inhibition of beating was observed with RS 30570 at the highest concentration tested (10^{-5} M).

Effects of RS 30026 on contractility of embryonic chick myocytes

The contractility of embryonic chick myocytes can be assessed by measuring edge movement of aggregates of myocytes, as described by Clusin (1981), and in this study edge movement and contractility have been assumed to be synonymous. Edge movement was recorded by a video tracking device which measures the movement of a discrete area of the aggregate,

Figure 2 Reversal of nisoldipine-induced inhibition of aggregate beating. Aggregates of myocytes were incubated with $0.3 \mu\text{M}$ nisoldipine throughout, a concentration which causes inhibition of beating in 85% of aggregates. Addition of increasing concentrations of Bay K 8644 (●) or RS 30026 (○) reversed nisoldipine-induced inhibition of beating. pEC_{50} values were 7.20 for Bay K 8644 ($n = 16$) and 7.45 for RS 30026 ($n = 12$). Vertical lines show s.e.mean.

chosen on the basis of it displaying most movement. Typical control responses were around 2–5 μm . The method used has been described in detail elsewhere (Patmore *et al.*, 1989a). Positive inotropic responses in embryonic chick myocytes can be difficult to measure, since in normal extracellular calcium concentrations of 2 mM the myocytes are near the top of the calcium-twitch contraction relationship (Patmore *et al.*, 1989a). Thus in order to observe positive inotropic responses to calcium facilitators the external calcium was reduced to 1 mM, which is around 50% of maximal. It has previously been shown that embryonic chick myocyte aggregates respond rapidly to positive inotropic agents (Patmore & Whiting, 1985; Patmore *et al.*, 1989b). The effects of RS 30026 on edge movement are shown in Figure 3 together with the concentration-response relationship. The concentration of RS 30026 which increased contractility by 50% above control levels (EC_{50}) was 2 nM which compares with 3 nM obtained for Bay K 8644 in similar experiments (Patmore *et al.*, 1989b). Whilst the potencies for initial positive inotropism for the two drugs were similar, RS 30026 caused a 5–6 fold increase in edge movement at 30 nM, compared with the 2 fold increase by Bay K 8644 obtained by Patmore *et al.* (1989b).

Effects on cardiac contractility (papillary muscle)

Concentrations of calcium facilitators which caused an increase in contractility of papillary muscle to 200% of control (pEC_{200} values) are shown in Table 2. Whilst pEC_{200} values for Bay K 8644 and RS 30124 were similar, RS 30026, the thiolactone derivative of RS 30124 was more than 10 times more potent. CGP 28392 displayed weak activity in these experiments. This increase in potency is shown in concentration-response relations for these compounds in Figure 4. Interestingly, the substitution of a sulphur for

Figure 3 Effects of RS 30026 on edge movement of aggregates of myocytes. Measurements of edge movement were made from single aggregates of myocytes which display automaticity. (a) Addition of 30 nM RS 30026 to the superfusate caused a large increase in edge movement or contractility with a substantial increase in aggregate 'tone'. In the presence of RS 30026 the natural rate is slower. (b) Quantification of the inotropic effects of RS 30026. Data are means for $n = 4$ determinations at each concentration; vertical bars show s.e.mean. The superfusate contained 1 mM calcium throughout to allow observation of positive inotropism.

Table 2 Effects of calcium channel agonists on cardiac and vascular smooth muscle

	Cardiac ventricular muscle (Guinea-pig papillary muscle) pEC_{200}	Coronary vascular smooth muscle (Porcine coronary artery rings) pEC_{50}
Bay K 8644	6.79	8.26
CGP 28392	5.18	7.43
RS 30124	7.00	7.61
RS 30026	8.21	8.88

Data shown are negative logarithms of concentrations causing a 100% increase (doubling) of contractility of guinea-pig papillary muscle and a 50% of maximum augmentation of 30 mM potassium-induced contracture of porcine coronary artery rings. Values were obtained from a least squares non-linear regression fit of a sigmoid function to data obtained from 4–9 experiments.

oxygen in the lactone group of this molecule increases the potency and the efficacy of the compound as a positive inotrope. At 10^{-6} M Bay K 8644 increased contractility to around $327 \pm 38\%$ ($n = 9$) of control whilst RS 30124 increased twitch magnitude to a mean value of $868 \pm 110\%$ ($n = 3$). RS 30026 increased contractility to $1318 \pm 498\%$ ($n = 4$) of control values (all values are mean $\pm$ s.e. of mean).

Effects on coronary artery

Potencies for constriction of 30 mM potassium depolarized coronary artery rings (pEC_{50} values) by Bay K 8644, CGP 28392, RS 30124 and RS 30026 are shown in Table 2. In these experiments RS 30026 was more potent than Bay K 8644 with EC_{50} values of 1.3 and 5.5 nM respectively, differences in the maximum contraction were not observed as the design of the experiment only allowed contraction of the rings to a maximal 100% contracture. Concentration-response relationships for Bay K 8644 and RS 30026 are compared in Figure 5. CGP 28392 was also more active in relation to Bay K 8644 than was observed in experiments on papillary muscle.

Effects on calcium channel current

Since reversal of nisoldipine-induced antagonism of aggregate beating appears to be specific to agents which have a direct action on the calcium channel and is insensitive to isoprenaline or to cardiac glycosides such as ouabain (Patmore *et al.*,

Figure 4 Effects of calcium channel agonists on contractility of guinea-pig papillary muscle. Positive inotropic responses to CGP 28392 (■, $n = 4$), Bay K 8644 (▲, $n = 9$), RS 30124 (●, $n = 3$) and RS 30026 (○, $n = 3-6$) were expressed as a percentage of the control contractility at the start of the experiment. Effects of each concentration were monitored over 30 min. Higher concentrations of Bay K 8644 caused no further increase in contractility whilst at 10^{-5} M CGP 28392 increased contractions to $220 \pm 12\%$ of control (mean $\pm$ s.e.mean, $n = 4$). Vertical lines show s.e.mean.

Figure 5 Effects of Bay K 8644 and RS 30026 on porcine coronary artery rings. Concentration-dependent facilitation of contractions of porcine left anterior descending coronary artery rings partially contracted with 30 mM K⁺ by Bay K 8644 (○, n = 4) and RS 30026 (●, n = 4–8). Vertical lines show s.e.mean.

1989b), it seems that the positive inotropic and vasoconstrictor properties of RS 30026 are a feature of calcium channel activation and or facilitation. To demonstrate this activity we have made direct recordings of the voltage-activated calcium channel currents in embryonic chick myocytes. RS 30026 (100–500 nM) and Bay K 8644 (50–500 nM) produced large increases in calcium channel current amplitude, with a larger transient component to the current (Figures 6 and 7). RS 30026 (100 nM, n = 3; and 500 nM, n = 4) shifted the voltage at which current was maximal from +10 mV to –10 mV (Figure 6). The threshold at which current was observed was also shifted by 10 mV in a hyperpolarising direction by RS 30026 (100–500 nM). Depolarizations to –30 mV elicited little or no current under control conditions, but in the presence of RS 30026 a slowly activating sustained current was observed (Figure 6). The effects of Bay K 8644 were examined in a separate series of experiments. Bay K 8644 (50–500 nM) increased calcium channel currents as expected (Figure 7), but the magnitude and dose-dependency of the effects were less than those of RS 30026, at the concentrations tested. From the voltage-current relationship the conductance (g) was calculated for each potential (V_m) from $g = I / (V_m - V_{rev})$ where V_{rev} is the reversal potential and I the peak inward current. These values are plotted against voltage in Figure 8. These activation curves have been fitted with a non-linear least squares procedure to a Boltzmann distribution where $g = g_{max} / (1 + \exp((V_m - V_{1/2})/k))$. Voltages at which the current was half activated are compared in Table 3. RS 30026 caused a shift in the voltage at which the calcium current was half-maximally activated to more negative values with a 12.6 mV shift at 500 nM. This was similar to the shift of 11 mV observed with 50 nM Bay K 8644. Interestingly at 500 nM Bay K 8644 the shift in the activation curve was very much smaller (1.9 mV), presumably reflecting the antagonist properties of Bay K 8644 at this concentration. A marked difference in the slope of the calcium current activation curves was observed between Bay K 8644 and RS 30026. With Bay K 8644, the slopes were similar in the presence and absence of the drug. RS 30026 caused a concentration-dependent increase in slope (decrease in k) reflecting the activation of more current for a given depolarization. On repolarization from potentials of

around 0 to +30 mV inward tail currents were usually observed. In the presence of 500 nM RS 30026 these became very large and slowly inactivating (Figure 6).

Discussion

Reversal of nisoldipine-induced inhibition of myocyte aggregate beating appears to be a useful and specific functional screen for identifying potential dihydropyridine calcium channel agonists. This assay is not sensitive to other positive inotropic agents such as isoprenaline or the cardiac glycoside strophanthin G (ouabain) (Patmore *et al.*, 1989b). Thus compounds which are positive in this test appear to have agonist

Figure 6 Effects of RS 30026 on whole-cell calcium channel currents in chick myocytes. Cells were held under voltage clamp at –80 mV and stepped to test potentials for 700 ms. (a) Current-voltage relationship for the peak inward current in the absence (□) and presence of 100 nM (▲) and 500 nM (●) RS 30026. Data are mean values from 4 experiments. (b) Representative current records evoked by depolarizations to test potentials as shown in the absence and presence (*) of 500 nM RS 30026. Note the very large tail currents on repolarization from 0 and +30 mV.

Table 3 Effects of Bay K 8644 and RS 30026 on activation of calcium currents in embryonic chick myocytes

	Control	Bay K 8644 50 nM	500 nM	Control	RS 30026 100 nM	500 nM
g _{max} (nS)	2.65	5.66	5.45	3.34	5.32	12.38
V _{0.5} (ΔV _{0.5})	–15.2 (0)	–26.2 (11)	–17.1 (1.9)	–9.0 (0)	–18.1 (9.1)	–21.6 (12.6)
k (mV)	6.28	6.74	6.11	8.10	4.19	3.86

Conductance data are from fits of a Boltzmann function as described in the text to mean conductance-voltage relationships for 4 experiments for each treatment. Values shown in parentheses are shifts in the half-activation voltage (V_{0.5}) from control.

Figure 7 Effects of Bay K 8644 on whole-cell calcium channel currents in chick myocytes. Cells were held under voltage clamp at -80 mV and stepped to test potentials for 700 ms. (a) Current-voltage relationship for the peak inward current in the absence ($\square$) and presence of 50 nM ($\blacktriangle$) and 500 nM ($\bullet$) Bay K 8644. Data are mean values from 4 experiments. (b) Representative current records evoked by depolarizations to test potentials as shown in the absence and presence (*) of 50 nM Bay K 8644.

characteristics. However, it might be considered that dihydropyridines which have reasonable affinity for the calcium channel but have low antagonist efficacy may displace nisoldipine and therefore appear positive in this test, but not necessarily be agonists at the calcium channel. In the series of dihydropyridine-lactone compounds described, we have evaluated several of the most potent for positive inotropic activity in guinea-pig papillary muscle (RS 30026 and 30124—Table 2; RS 31227, 30321, 34667 and 31156—data not shown). All these compounds were positively inotropic and thus appear to be agonists.

The dihydropyridine-type calcium channel activators described so far, such as the 3-nitro derivatives Bay K 8644 (Schramm *et al.*, 1983a) and 202-791 (Hof *et al.*, 1985), the lactone CGP 28392 (Erne *et al.*, 1984), the 2-amino derivative H 160/51 (Beyer *et al.*, 1985), and the amide YC-170 (Takenaka & Maeno, 1982) constitute a structurally heterogeneous group. Theoretical calculations (Mahmoudian & Richards, 1986) and comparisons between crystal structures of two of these agonists, Bay K 8644 and CGP 28392, with those of known antagonists, have led to proposals explaining the conformational differences between agonists and antagonists in terms of hydrogen bonding of the 1-NH group, ester group orientation and hydrophobic fit (Langs & Triggle, 1985). It is, however, not clear how structures such as YC-170 or H 160/51 fit into this picture. Furthermore, our own observation that optically inactive dihydropyridines with hydrogen atoms instead of methyl groups in the 2- and 6-positions also displayed agonist activity (R. Greenhouse, unpublished work) complicates this issue even further. In the present series, no clear-cut structure-activity relationship emerged apart from the observation that bulky, lipophilic substituents on the 4-phenyl group generally increased facilitator activity. Additionally, replacing the lactone oxygen with sulphur, as in RS-30026 and RS 34389, resulted in an enhancement of agonist activity.

Figure 8 Effects of Bay K 8644 (a) and RS 30026 (b) on calcium channel current voltage-conductance relationships. Conductances (g) were calculated from the relation: $g = I_{\text{peak}} / (V_m - V_{\text{rev}})$ where the reversal potential (V_{rev}) for the calcium channel current was 60 mV from current-voltage data shown in Figures 6 and 7. Curves fitted to the data describe the Boltzmann function where $g = g_{\text{max}} / (1 + \exp((V_m - V_{1/2})/k))$. Values of g_{max} , $V_{1/2}$ and k are shown in Table 3. (a) Control ($\square$) conductance-voltage relationship and in the presence of 50 nM ($\blacktriangle$) and 500 nM ($\bullet$) Bay K 8644. (b) Control ($\square$) and in the presence of 100 nM ($\blacktriangle$) and 500 nM ($\bullet$) RS 30026.

A functional consequence of reversal of calcium antagonist-induced inhibition of aggregate beating is that the compounds should be positively inotropic in these myocytes. This activity has been demonstrated with the most potent compound from this series, RS 30026. In previous studies positive inotropism has only been observed when the calcium concentration was reduced to 1 mM (50% the normal concentration), since at 2 mM the contractility of aggregates is very close to the top of the external calcium response curve (Patmore *et al.*, 1989a,b). Under these conditions the standard calcium channel facilitator Bay K 8644 only increased contractions up to the level attained in 2 mM calcium (i.e. a 2 fold increase in contractility or edge movement). The effects of RS 30026 were more marked; whilst the EC_{50} (2 nM) was similar to that for Bay K 8644 (3 nM), RS 30026 increased edge movement to 5–6 times the control level in 1 mM. These data demonstrate that RS 30026 is a more effective calcium channel agonist than Bay K 8644.

In guinea-pig papillary muscle the effects of RS 30026 were even more marked. In this preparation Bay K 8644 caused considerable positive inotropism, increasing the magnitude of twitch contractions to 350% of control values at $1 \mu\text{M}$, with an EC_{200} of $0.16 \mu\text{M}$. At higher concentrations ($10 \mu\text{M}$) there was a reduction in the positive inotropic effect presumably indicating evidence of calcium channel antagonism. The RS 30026 series was based on the compound CGP 28392 which is a less potent and weaker agonist than Bay K 8644 (Rogg *et al.*, 1985; Truog *et al.*, 1985; Patmore & Duncan, 1988). CGP 28392 displayed less positive inotropic activity than Bay K 8644, only increasing contractility up to 220% of control at $10 \mu\text{M}$, with an EC_{200} of $6.6 \mu\text{M}$. RS 30124 is directly related to CGP 28392, both compounds are lactone-dihydropyridines with OCH_2Ph replacing the OCHF_2 group of CGP 28392.

The significance of this modification is demonstrated in Figure 4, RS 30124 increased contractility up to 868% of control levels whilst the EC_{200} of $0.1 \mu M$ was similar to that for Bay K 8644. Replacing the lactone oxygen atom with sulphur (RS 30026) makes a significant difference to the positive inotropic actions of the molecule. RS 30026 increased contractility up to 1318% of control levels at $1 \mu M$ with an EC_{200} of $6 nM$; it was 26 times more potent than Bay K 8644 and over 3 times more effective in potentiating the magnitude of contractions.

Differences between the effectiveness of these compounds in potentiating contractions of $30 mM$ K^+ depolarized porcine coronary artery rings were not so obvious (Table 2). CGP 28392 was more potent in this assay ($EC_{50} = 37 nM$) and RS 30124 displayed a similar potency ($EC_{50} = 24 nM$). Bay K 8644 was more potent than RS 30124, with an EC_{50} of $5.5 nM$, and RS 30026 was 4 times more potent than Bay K 8644, with an EC_{50} of $1.3 nM$. It was not possible to observe differences in the magnitude of contraction in this preparation since rings were only able to constrict to a maximum 100% level. Whilst the compounds showed a similar rank order of potency as vasoconstrictors, the differences were not so marked as those observed in cardiac muscle. This may be attributed to the different state of the calcium channels in this preparation, since in $30 mM$ potassium the membrane potential will be considerably depolarized (around -20 to $30 mV$ as estimated from the Nernst equation) and this is considerably different from the $-85 mV$ resting potential observed in papillary muscle preparations.

To demonstrate that RS 30026 does facilitate calcium currents in myocytes, direct measurement of the calcium channel currents which underlie contractility was made with the whole-cell voltage clamp technique. RS 30026 at 100 and $500 nM$ caused marked increases in the magnitude of the calcium channel current, and produced a shift in the threshold for activation of current and a negative ($-20 mV$) shift of the peak current potential. Similar but smaller increases in current magnitude were observed with Bay K 8644 and these were maximal at $50 nM$, a higher concentration of $500 nM$ caused a smaller increase in the peak current. Bay K 8644, at $50 nM$, also shifted the threshold for activation of currents to more negative potentials and caused a $-10 mV$ shift in the potential at which the current was maximal. These observations were quantified in an analysis of the conductance-voltage relationships, which were fitted to a Boltzmann distribution describing current activation with depolarization. The activation characteristics of the calcium channel currents of embryonic chick myocytes are similar to those observed in adult guinea-pig myocytes. In embryonic chick myocytes half-maximal activation occurred at -15 to $-9 mV$ with a slope (k) of $6-8 mV$ for an e fold increase in conductance. This compares with $-14.9 mV$ (range = -25 to $-5 mV$), $k = 6.2$ for guinea-pig myocytes found by Kamp *et al.* (1989). Bay K 8644 ($50 nM$) caused a parallel shift of the activation curve by $-11 mV$. Similar parallel displacements have been obtained by Hamilton *et al.* (1987) ($-12 mV$ for $1 \mu M$ (+)-Bay K 8644) in guinea-pig myocytes. The threshold for conductance activation was also shifted to more negative potentials in the presence of RS 30026, but the most noticeable effect was on the slope of the conductance-voltage relationship, with k decreasing from $8.1 mV$ to $4.2 mV$ in $100 nM$ and to 3.9 in $500 nM$. Thus the shift of the activation curve in the presence of RS 30026 was not parallel and in this respect the action of RS 30026 is different from Bay K 8644 and the agonist isomer of the dihydropyridine (+)-202-791, which has been shown by Kamp *et al.* (1989) to shift the activation curve by $-10.5 mV$ at $400 nM$, whilst k values were similar in the presence ($k = 5.41$) and absence ($k = 6.17$) of the drug. It is possible

that an increase in the slope may be a function of uncompensated series resistance of the recording pipette, giving rise to larger voltage steps than the command potentials when the magnitude of the current was increased with RS 30026. At the worst case where the conductance is maximal the current increases from $140 pA$ at $+20 mV$ to $740 pA$ at $0 mV$, this would lead to a $3.6 mV$ shift of the voltage based on an estimated series resistance of $6 M\Omega$ (see Methods). At the half activation voltage the shift due to uncompensated series resistance would be only 0.4 compared with the $9.1 mV$ observed. Thus errors associated with series resistance artefacts would be small compared to the shifts in conductance observed in the presence of RS 30026. It may be considered that the series resistance could be higher than our estimate, but with similar increases in current in the presence of Bay K 8644 no changes in slope were observed.

These observations indicate that as the membrane potential depolarizes, the activation of the calcium channel conductance will be more rapid in the presence of RS 30026 than in the presence of Bay K 8644 or (+)-202-791, and that RS 30026 has different effects to other dihydropyridine agonists on the gating of the calcium channel. However, it does not seem likely that differences in activation characteristics can entirely account for the very large increases in contractility observed with RS 30026. Evidence to support this view comes from the very slowly decaying tail currents observed with RS 30026. These enhanced tails were also observed to a lesser extent in the presence of Bay K 8644, and are a reflection of the increased open time of the channel so that channels remain open after the membrane potential has been repolarized. The magnitude of the tail currents in the presence of RS 30026 indicates that this compound has marked effects on channel open time and it will be of obvious interest to investigate these actions at the single channel level. Thus these two phenomena of increased activation of the calcium current and marked prolongation of channel open time would result in a considerable increase in calcium influx in myocardial cells and can explain the extreme positive inotropism observed with RS 30026.

Throughout these studies we have used the racemic forms of Bay K 8644 and RS 30026. Previous work has shown that the individual enantiomers of Bay K 8644 (Frankowiak *et al.*, 1985), 202-791 (Hof *et al.*, 1985) and H 160/51 (Beyer *et al.*, 1985) have opposite effects on the calcium channel; one isomer being an antagonist and the other an agonist. If the same is true with RS 30026 then the agonist isomer may be even more potent and effective than we have demonstrated here for the racemate. In the present experiments high concentrations of Bay K 8644 showed antagonist properties, which may be a reflection of the actions of the (+)-antagonist isomer or, as suggested by MacDonald *et al.* (1987), may also reflect antagonism by high concentrations of the (-)-agonist isomer. No such effects were observed with the racemic RS 30026 and we have described considerable differences in the efficacy of Bay K 8644 and RS 30026. This may indicate that either the isomers of RS 30026 are both agonists or the antagonist isomer is less effective than the (+)-antagonist isomer of Bay K 8644 or, perhaps, there are significant differences in the intrinsic efficacy of the agonist isomers of the two agents, with Bay K 8644 behaving as a partial agonist and RS 30026 as a full agonist. The resolution of the isomers of RS 30026 is currently being undertaken and we will shortly be able to address these possibilities.

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