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Use of D-myo inositol 1,2,6 trisphosphate to inhibit contractile activity in rat ventricular cardiomyocytes induced by neuropeptide Y and other cardioactive peptides through phospholipase C

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1 D-Myo inositol 1,2,6 trisphosphate (α-trinositol, pp56), an isomer of the second messenger substance, inositol 1,4,5 trisphosphate, has an interesting pharmacological profile that includes antagonism of a number of neuropeptide Y (NPY)-mediated cellular processes. The ability of pp56 to inhibit selectively the myocardial contraction mediated by NPY in relation to the responses to other cardioactive peptides, including endothelin-1, calcitonin gene-related peptide (CGRP), secretin and vasoactive intestinal peptide (VIP), was assessed. In order to investigate the possible interaction of pp56 with mechanisms of inositol phosphate signalling generated in heart muscle cells by activation of the β-isoenzyme of phospholipase C (PLCβ), noradrenaline was used as a positive control, and isoprenaline and forskolin were included as negative controls.

2 Ventricular cardiomyocytes, isolated from the hearts of adult rats, were stimulated to contract at 0.5 Hz in the presence of calcium ion (2 mM). The concentrations of agonists used were in the region of their maximally effective concentrations for myocyte contraction and the concentration of pp56 was in the range of 1–100 μM. Contractile activity was monitored by video microscopy and maximum shortening determined by image analysis.

3 In the absence of agonist, contractile amplitudes following 20 min preincubation with pp56 were not different from that observed in the absence of pp56. Pp56 (1–100 μM) inhibited significantly the positive contractile response to noradrenaline (5 μM) in the presence of propranolol (500 nM), such that the response was almost completely attenuated at the highest concentration of the inhibitor. Pp56 did not inhibit the positive contractile responses to forskolin (40 μM) or isoprenaline (100 nM).

4 NPY alone does not influence the basal level of contraction of cardiomyocytes, but can attenuate isoprenaline-stimulated contraction and can increase contractile amplitude from basal when the transient outward current is blocked with 4-aminopyridine. In the presence of isoprenaline (100 nM), the negative response to NPY (100 nM) was attenuated significantly by pp56 (1–100 μM). With 4-aminopyridine, the positive contractile response to NPY (200 nM) was decreased by pp56, although this was not statistically significant.

5 Pp56 inhibited the positive contractile responses to CGRP (1 nM) and endothelin-1 (20 nM) completely, but did not affect the responses to secretin (20 nM) or VIP (20 nM).

6 In conclusion, these data challenge the previously obtained selectivity of pp56 as an antagonist of NPY-mediated cellular processes, since responses to CGRP and endothelin-1 were at least equally sensitive. Furthermore, as pp56 discriminated clearly in its inhibition of responses to α-adrenoceptor by comparison with β-adrenoceptor/adenylate cyclase stimulation, it appears that pp56 may be a useful pharmacological agent with which to distinguish between PLCβ-dependent and PLCβ-independent coupling mechanisms. On this basis, further evidence has been obtained that, in rat cardiomyocytes, the contractile responses to NPY, CGRP and endothelin-1 are attributable to the activation of PLCβ-dependent pathways, whereas the responses to secretin and VIP are mediated by PLCβ-independent pathways.

Keywords: D-Myo inositol 1,2,6 trisphosphate; pp56; neuropeptide Y; endothelin-1; calcitonin gene-related peptide; vasoactive intestinal peptide; phospholipase C; rat ventricular cardiomyocytes; contractile function

Introduction

D-Myo inositol 1,2,6 trisphosphate (α-trinositol, pp56), an isomer of the second messenger substance, D-myo inositol 1,4,5 trisphosphate, does not occur naturally in mammalian tissues, but has been shown to have an interesting pharmacological profile, including inhibition of the direct vasoconstrictor effects of neuropeptide Y (NPY) and potentiation by NPY of noradrenaline-mediated vasoconstriction *in vivo* (Sun *et al*., 1991; 1992; 1994) and *in vitro* (Edvinsson *et al*., 1990; Adamsson *et al*., 1992; Ralevic *et al*., 1994). The ability of pp56 to attenuate selectively the effects of NPY, without reducing the responses to several other vasoconstrictor stimuli, led Edvinsson and co-workers to conclude that pp56 is a relatively selective antagonist of NPY-mediated cellular processes (Edvinsson *et al*., 1990), although this finding is not unanimous (Pernow *et al*., 1992; Feth *et al*., 1993). A direct or allosteric interaction of pp56 at receptors for NPY is uncertain (Wahlestedt *et al*., 1992; Feth *et al*., 1993) and it is more probable that pp56 might interfere with a signalling mechanism utilized by NPY receptors.

Neuropeptide Y (NPY) can both decrease and increase the contractile response of electrically-stimulated rat ventri-

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cular cardiomyocytes and various signalling pathways have been identified (Millar *et al*., 1991). The negative contractile effect, observed in isoprenaline-treated cells, is thought to be due primarily to stimulation of the transient outward current. Inhibition of isoprenaline-stimulated accumulation of cyclic AMP (Piper *et al*., 1989) and attenuation of the accumulation of inositol 1,4,5 trisphosphate by NPY have also been demonstrated (Xiang & Brown, 1993), although the relevance of these mechanisms to NPY receptor-mediated contractile coupling is uncertain. NPY alone does not influence the basal level of contraction of cardiomyocytes, but in the presence of 4-aminopyridine, which inhibits selectively the transient outward current in these cells, a positive contractile response to NPY is unmasked which has been attributed to influx of Ca^{2+} via L-type calcium channels (Millar *et al*., 1991).

In the control of cardiac contractility, it has been established that activation of α_1-adrenoceptors generates the signal through phospholipase C, specifically the β-isoenzyme (PLCβ) (reviewed by Terzic *et al*., 1993), whereas activation of β-adrenoceptors results in stimulation of the adenylate cyclase pathway (reviewed by Brodde *et al*., 1995), and these mechanisms have been demonstrated clearly at the level of the myocardial cells. The positive contractile effect elicited by activation of α-adrenoceptors, specifically of the α_{1A}-subtype, in ventricular cardiomyocytes is associated with the accumulation of inositol 1,4,5 trisphosphate (Buxton & Doggweiler, 1988), elevation in intracellular Ca^{2+} concentration (Eckel *et al*., 1991) and activation of protein kinase C (Iwakura *et al*., 1990; Kaku *et al*., 1991). In contrast, the positive contractile effects of isoprenaline, a β-adrenoceptor agonist, and forskolin, a direct activator of adenylate cyclase, are associated with the accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Kameyama *et al*., 1985; Kelso *et al*., 1993) and activation of cyclic AMP-dependent protein kinase (Li & Sperelakis, 1983; Hartzell *et al*., 1991).

In addition to NPY, various other peptide substances also have significant inotropic effects and the signal transduction mechanisms have been investigated to a limited extent in rat ventricular cardiomyocytes. Secretin and VIP are potent stimulants of cyclic AMP (Bell & McDermott, 1994a), and the positive contractile responses to these agonists are attenuated markedly by the cyclic AMP antagonist, Rp-adenosine cyclic 3′:5′-phosphorothioate (Rp-cAMPS) (Bell & McDermott, 1994b). In comparison, the positive contractile response to calcitonin gene-related peptide (CGRP) is not associated with the accumulation of cyclic AMP (Bell & McDermott, 1994c) and is not attenuated by Rp-cAMPS (Bell & McDermott, 1994b); since CGRP stimulates the activation of protein kinase C in these cells, receptors for this peptide may couple to the regulation of PLCβ in rat ventricular cardiomyocytes (Bell *et al*., 1995; Bell & McDermott, 1996). Endothelin-1 exerts a positive contractile response and accumulation of multiple second messenger substances, including inositol 1,4,5 trisphosphate, has been demonstrated (reviewed in de Jonge *et al*., 1995).

The initial purpose of this study was to assess the use of pp56 as a selective inhibitor of NPY-mediated responses in the myocyte contraction bioassay. Since the mechanism is likely to be a functional interaction and because of the structural similarity between pp56 and inositol 1,4,5 trisphosphate, it was hypothesized that pp56 may interfere with mechanisms that are dependent on the activation of PLCβ. This was investigated by the application of appropriate controls, including the α-adrenoceptor agonist, noradrenaline (with propranolol added to block the small β-adrenoceptor-mediated effect), which acts by a mechanism dependent, at least in part, upon the stimulation of PLCβ, and forskolin and isoprenaline, which act by PLCβ-independent processes. Finally, the effects of pp56 on contractile responses to endothelin-1, CGRP, secretin and VIP were examined for their evidence in relation to the associated contractile coupling mechanisms.

Methods

Isolation of cardiomyocytes

Rat ventricular cardiomyocytes were isolated according to methods originally described by Piper *et al*. (1982). Briefly, 12-week-old male Sprague-Dawley rats were subjected to deep isoflurane anaesthesia. The hearts were excised and perfused retrogradely with a Langendorff perfusion apparatus with a Ca^{2+}-free Krebs-Ringer solution containing collagenase (400 μg ml^{-1}) until they became flaccid. The hearts were then chopped finely and the mince was agitated gently in the same solution to dissociate individual cells. The resulting cell suspension was filtered to remove undigested material and the cells were sedimented at 500 r.p.m. for 3 min. Calcium-tolerance of the cells was restored gently by resuspending the sediment in Krebs-Ringer solution containing progressively higher concentrations of Ca^{2+} to a final concentration of 1 mM. The cell suspension was then applied to a 4% (w/v) albumin solution in order to sediment viable cardiomyocytes and effectively remove non-muscle cells and cell debris. The resultant sediment was resuspended in simple HEPES-based solution.

Experimental protocols

For the assessment of cellular contractile response, cells were resuspended at a concentration of 1×10^5 viable cardiomyocytes ml^{-1}. Aliquots (800 μl) were pipetted gently onto 4% (v/v) FCS-coated Petri dishes and incubated for 2 h, by which time viable cardiomyocytes had attached selectively to the surface of each dish. The dishes were washed with simple HEPES-based solution to remove non-attached cells and cell debris, and attached cells were incubated for a specified period (0–35 min) in simple HEPES-based solution (800 μl), in the absence or presence of pp56 (1–100 μM). This solution was subsequently removed and the cells were incubated for a further 3 min in modified HEPES-based solution (2.5 ml) containing in each experiment an identical concentration of pp56 to that present during the period of pre-incubation and a maximally-effective concentration of agonist and/or an appropriate concentration of 4-aminopyridine or propranolol, as stated in Table 1. The temporal-dependence and concentration-dependence of the contractile response to each agonist had been determined in previous studies (Millar *et al*., 1991; Bell & McDermott, 1994a, b, c).

Measurement of cellular contraction

At the end of this second period of incubation, each Petri dish was placed on a microscope stage thermoregulated at 37°C. With the microscope visual field in between, two silver chloride electrodes 6 mm apart were immersed to a distance of 5 mm into the fluid. Biphasic electrical stimuli composed of two equal but opposite rectangular 50 V stimuli of 0.5 ms duration were applied at a frequency of 0.5 Hz for 2 min. Images each including 4–8 individual cells were visualized at a magnification of 500 fold on a video monitor screen. The cells to be investigated were selected by use of a mouse, by marking points just beyond each end of the longitudinal axis of the cell. A straight line was interpolated between these two points and cell length was determined by edge detection using the software, HEARTBEAT, developed by the Parallel Processing Unit, Department of Computer Science, The Queen's University of Belfast, U.K. A transputer-based Parsys Supernode and Meiko Computing Surface multiprocessor computers were used to perform the computations. The sequence of 25 frames during which the contractile event had occurred was analysed one frame at a time. The data file created was inspected visually and the maximum diastolic length (A), which was the most commonly occurring length within each group of 25 frames, and the fully contracted, systolic, length of each cell (B) contracting in synchrony with the electrical stimuli, were

obtained from these data. The contractile response (dL) was expressed as the percentage (A-B) × 100/A.

Materials

CGRP (human α), secretin (porcine), VIP (porcine) and NPY (porcine) were obtained from Bachem Feinchemikalien A.G. (Bubendorf, Switzerland). Endothelin-1 (human) was purchased from Bachem California (U.S.A.). D-Myo inositol 1,2,6 trisphosphate (pp56) was a gift from Perstorp Pharma (Lund, Sweden). Noradrenaline hydrochloride and (±)-propanolol hydrochloride were obtained from Research Biochemicals Incorporated (Natick, U.S.A.). Polyethyleneimine (50% v/v), foetal calf serum (FCS), bovine serum albumin (cat. no A7030), (±)-isoprenaline hydrochloride, and forskolin were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, U.K.). 4-Aminopyridine was supplied by Aldrich Chemical Company Ltd. (Gillingham, Dorset, U.K.). Collagenase prepared from *Clostridium histolyticum* strain C (Cat. No. 17449, 0.92 u ml^{-1}) was purchased from Serva Feinbiochemica (Heidelberg, Germany). Petri dishes (35 mm diameter) were supplied by Nunc (Denmark). All other chemicals used were of analar grade and purchased from BDH Chemicals Ltd. (U.K.).

The composition of the calcium-free Krebs-Ringer solution was as follows (mM): NaCl 110, KCl 2.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11. This solution was gassed with 95% O_2/5% CO_2 and maintained at a pH of 7.4 at 37°C. The composition of the 'simple HEPES-based solution' was as follows (mM): NaCl 125, KH_2PO_4 1.2, KCl 2.6, $MgSO_4$ 1.2, HEPES free acid 10, $CaCl_2$ 1 and glucose 11; pH 7.4. The composition of the 'modified HEPES-based solution' was as follows (mM): NaCl 125, KH_2PO_4 1.2, KCl 4.6, $MgSO_4$ 1.2, HEPES free acid 10, $CaCl_2$ 2 and glucose 11; pH 7.4.

Eppendorff tubes were treated with 1% (v/v) polyethyleneimine solution, in order to reduce non-specific adherence of peptide to the walls of the tubes. Peptides were reconstituted in simple HEPES-based solution containing 0.2% (w/v) bovine serum albumin and aliquots were stored at −20°C. Pp56 was reconstituted in simple HEPES-based solution and aliquots of the stock solution (0.1 M) were stored at −20°C. Solutions of isoprenaline hydrochloride, propanolol hydrochloride, noradrenaline hydrochloride and 4-aminopyridine were prepared daily. Solutions of 4-aminopyridine were kept in the dark until immediately before use. Petri dishes were coated with 4% (v/v) FCS in simple HEPES-based solution.

Statistical analysis

Under each experimental condition, 20 cardiomyocytes from each heart cell population were measured and the average value was obtained. Data of contractile response are given as mean values ± s.e.mean of 5 heart cell preparations. Analysis of the differences between responses obtained in the presence of pp56 and under control conditions was performed by a one-way analysis of variance (SPSS-PC) and *post hoc* analysis carried out with a multiple range test (Scheffe). A paired Student's *t* test was used to test differences in contractile responses obtained if appropriate under different conditions in the presence of the same concentration of pp56, and also between responses at the beginning and end of experiments. Differences with $P<0.05$ were regarded as significant.

Results

In suspensions of freshly isolated cardiomyocytes, the percentage of viable cells was, typically, greater than 65%. No deleterious effects upon cellular viability, such as hypercontraction and subsequent rounding of previously viable cells, or increased incidence of membrane blebbing or spontaneous contraction, were observed following incubation for 1 h with pp56 (1 mM) (data not shown). The coefficient of assay variation, determined by measuring amplitudes of cellular contraction during 2 min of electrical stimulation following 3 min of pre-incubation in modified HEPES-based solution, in four groups, each attached to different Petri dishes, of 20 cardiomyocytes derived from the same heart cell population was 3.5% (intra-assay variation, $n=4$), and of 20 cardiomyocytes in each of four groups derived from different preparations was 5.2% (inter-assay variation, $n=4$).

The temporal-dependence of the effect of pp56 was examined with noradrenaline as the agonist. As shown in Figure 1, pp56 did not have any effect on the basal contraction over the period of the experiment (35 min). However, in the presence of pp56 the response to noradrenaline was inhibited at times ⩾20 min. In subsequent experiments, therefore, a time of 20 min was chosen for pre-incubation with pp56, before application of the agonists.

For assessment of the effect of pp56 on contractile amplitude under basal conditions, analysis of the experimental data pooled from all experiments indicated that in the absence of pp56, the response obtained in the absence of agonist at the end of each experimental sequence (approximately 2 h later) (10.24 ± 0.29%, mean ± s.e.mean, $n=34$) declined significantly from that obtained at the beginning (10.98 ± 0.32%, mean ± s.e.mean, $n=34$), so that there was an apparent attenuation of contractile amplitude by pp56 at concentrations of 10 and 100 μM (Figure 2). Therefore, in order to eliminate the run-down of contractile response from analysis of the effects of pp56 *per se* or on the response to each agonist, time-paired data were obtained under each experimental condition and differences were calculated, as given in Table 1.

When the data were analysed in this way, the contractile amplitude obtained at the end of each experiment did not differ significantly from that obtained at the beginning; also, pp56 did not have any effect on the basal response. In investigations of the effects of the compound on PLCβ-dependent and PLCβ-independent agonist-stimulated responses, pp56 inhibited the

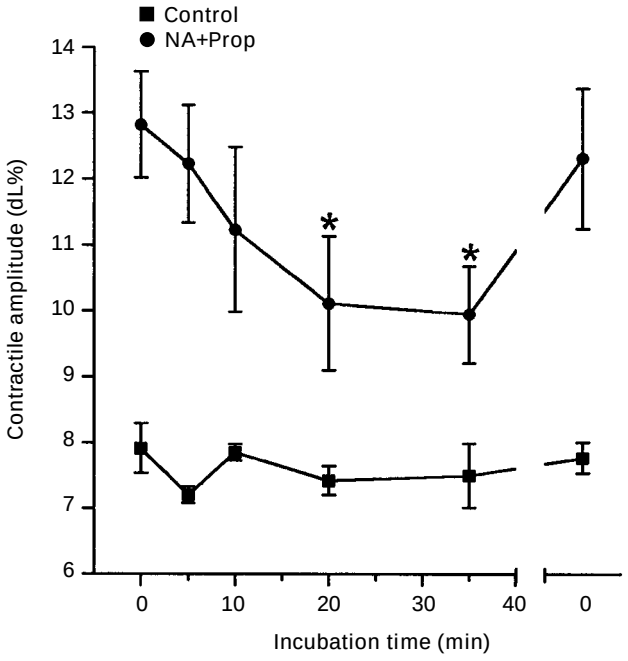

Figure 1 Temporal-dependence of the effects of pp56 (100 μM) under basal conditions or on the stimulation of contractile amplitude by noradrenaline. Cardiomyocytes were pre-incubated for 0–35 min in simple HEPES-based solution, supplemented with pp56, and then incubated for a further 3 min in modified HEPES-based solution alone (Control) or with the addition of noradrenaline (5 μM) and propanolol (500 nM) (NA + Prop), before stimulation at 0.5 Hz for 2 min. Control responses were elicited at the beginning and end of each experiment. Contractile responses are expressed as maximum shortening as a percentage of prestimulus length (dL%). Data are given as mean values of 5 heart cell preparations; vertical lines show s.e.mean. *Denotes significant variation from first control response. ($P<0.05$).

positive contractile response to noradrenaline (in the presence of propranolol), such that the response was almost completely attenuated at the highest concentration of the inhibitor, whereas pp56 did not inhibit the positive contractile responses to forskolin or isoprenaline.

Pp56 had marked effects on the contractile responses to NPY. In the presence of isoprenaline, the negative response to NPY was attenuated completely by pp56. 4-Aminopyridine, which inhibits the transient outward current, produced a small positive contractile response *per se*, which was not influenced by pp56. The positive contractile response to NPY, observed in the presence of 4-aminopyridine, was decreased by pp56, although this was not statistically significant. However, at the maximum inhibition obtained with pp56 (10 μM), the response in the presence of NPY was not different to that observed with 4-aminopyridine alone. The positive contractile responses to CGRP and endothelin-1 were attenuated completely by pp56. In contrast, the compound did not inhibit the positive contractile responses to secretin and VIP.

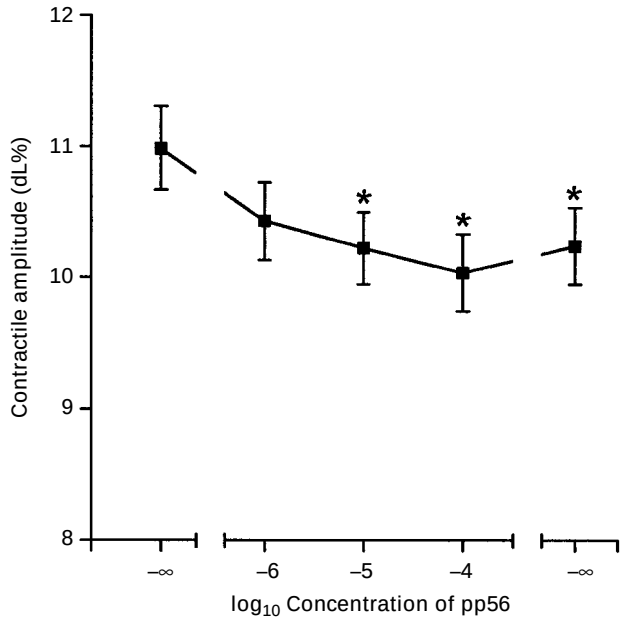

Figure 2 Concentration-dependence of the effect of pp56 upon amplitudes of cellular contraction elicited in the absence of agonist. Cardiomyocytes were pre-incubated for 20 min in simple HEPES-based solution supplemented with pp56 (1–100 μM), and then incubated for a further 3 min in modified HEPES-based solution supplemented with the same concentration of pp56, before electrical stimulation. Other details are as given in Figure 1. Data are given as mean values of 34 heart cell preparations; vertical lines show s.e.mean. *Denotes significant variation from first control response ($P<0.05$).

Discussion and conclusions

There has been considerable experimental evidence obtained in a range of vascular models *in vitro* and *in vivo* to indicate that antagonism by pp56 is selective for NPY responses (Edvinsson *et al*., 1990; Sun *et al*., 1991; 1994; 1995; Adamsson *et al*., 1992). However, there is also evidence that pp56 may not be as selective for antagonism of responses to NPY as found previously. Pp56 was found to inhibit the pressor responses to ATP and α-adrenoceptor agonists in normotensive pithed rats (Sun *et al*., 1991; 1992) and interfered with the actions of exogenous noradrenaline and sympathetic cervical trunk stimulation on thyroidal blood flow (Dey *et al*., 1993). Also, pp56 antagonized the vasoconstriction of basilar arteries of guinea pigs and influx of ^{45}Ca into rat vena cava vascular smooth muscle cells in response to ATP (Wahlestedt *et al*., 1992) and inhibited thrombin-induced aggregation of platelets from healthy human volunteers (Winther *et al*., 1989). However, many of these attenuating effects occurred at higher concentrations than those required for inhibition of NPY-mediated responses, which may explain the nonspecificity of the inhibition by pp56 in these systems.

In this study, although pp56 attenuated the negative contractile response to NPY observed in isoprenaline-stimulated cardiomyocytes, and also the positive contractile response to NPY unmasked in the presence of 4-aminopyridine, the compound also inhibited the positive contractile responses to endothelin-1, CGRP and the α-adrenoceptor agonist, noradrenaline. Again it could be argued that pp56 was non-selective at the concentrations (1–100 μM) at which inhibitory effects were observed in rat ventricular cardiomyocytes, since a much smaller concentration (10 pM) antagonized the vasoconstrictor responses to NPY in isolated blood vessels while having no effect on vasoconstriction induced by phenylephrine or endothelin-1, or on vasorelaxation produced by CGRP (Edvinsson *et al*., 1990; Adamsson *et al*., 1992). However, in cardiomyocytes incubated with pp56 (1 μM), only partial inhibition of both the positive and negative contractile responses

Table 1 Effects of pp56 on contractile responses to inotropic agents in rat ventricular cardiac myocytes

		Contractile amplitude (ΔdL) *Concentration of pp56*			
Inotropic agent	*Control* (1)	1 μM	10 μM	100 μM	*Control* (2)
(Basal)		−0.4±0.01	−0.5±0.01	−0.5±0.01	−0.7±0.29
Noradrenaline (5 μM)+propranolol (500 nM)	3.2±0.29	1.4±0.77*	0.9±0.56*	0.5±0.34*	3.2±0.35
Forskolin (40 μM)	7.1±1.10	6.2±0.74	5.8±1.06	7.6±0.52	6.2±1.12
Isoprenaline (100 nM)	2.6±0.24	3.1±0.25	3.3±0.31	3.1±0.56	2.6±0.35
Isoprenaline (100 nM)+NPY (100 nM)	−0.3±0.55	1.1±0.62*	3.0±0.33*	2.3±0.70*	0.6±0.30
4-Aminopyridine (500 μM)	1.4±0.26	1.6±0.40	1.3±0.28	1.5±0.34	1.5±0.22
4-Aminopyridine (500 μM)+NPY (200 nM)	3.2±0.34	2.5±0.50	1.8±0.25	2.2±0.20	2.5±0.33
CGRP (1 nM)	2.1±0.63	−0.5±0.40*	0.0±0.33*	−0.3±0.66*	1.8±0.23
Endothelin-1 (20 nM)	1.6±0.29	−0.2±0.21*	−0.4±0.43*	−0.5±0.72*	1.9±0.24
Secretin (20 nM)	3.2±0.38	4.0±0.42	3.5±0.49	3.6±0.70	3.6±0.50
VIP (20 nM)	1.7±0.36	2.7±0.28	2.0±0.43	2.2±0.29	1.8±0.29

Cardiomyocytes were pre-incubated for 20 min in simple HEPES-based solution supplemented with pp56, and then incubated for a further 3 min in modified HEPES-based solution supplemented with the same concentration of pp56 alone or with the addition of inotropic agents at their maximally effective concentrations, before stimulation at 0.5 Hz for 2 min. Maximum diastolic shortening (dL) was calculated as a percentage of prestimulus length and data are expressed as differences (ΔdL) between the contractile amplitude elicited in the presence of pp56 alone and the time-paired response without pp56, or in the presence of pp56 with a given agonist and the time-paired response obtained in the absence of the agonist. Control (1) and Control (2) are the responses obtained in the absence of pp56 at the beginning and end of an experiment, respectively. Data are given as mean values±s.e.mean of 5 heart cell preparations. *$P<0.05$ denotes a significant difference with respect to Control (1).

to NPY was observed, while the positive contractile responses to CGRP and endothelin-1 were abolished completely, which indicates that these stimuli were at least as sensitive as NPY to the inhibitory effects of pp56.

It is unlikely that pp56 interferes directly or allosterically with NPY receptor-ligand interactions, since pp56 did not inhibit the binding of [^{125}I]-NPY to populations of NPY_1 receptors present on HEL and SK-N-MC cells, or to the population of NPY_2 receptors present on porcine splenic membranes (Feth *et al*., 1993) or the binding of [^{125}I]-PYY to rat vena cava smooth muscle cells *in vitro* (Wahlestadt *et al*., 1992). This conclusion is further supported by the observation that the antagonistic effects of pp56 do not occur on acute addition but require a pre-incubation interval of at least 20 min, as shown in this study in cardiomyocytes and also previously in vascular tissue (Edvinsson *et al*., 1990). It is more probable that pp56 may interfere as a functional antagonist of a signalling mechanism utilized by NPY receptors. Receptors for this peptide are coupled to the regulation of second messenger substances such as cyclic AMP and intracellular Ca^{2+} (reviewed in McDermott *et al*., 1993). However, pp56 did not antagonize either the NPY_1 receptor-mediated inhibition of forskolin-stimulated accumulation of cyclic AMP in HEL cells, NPY_1 receptor-mediated increases in intracellular Ca^{2+} concentration in HEL and SK-N-MC cells or NPY_3 receptor-mediated increases in intracellular Ca^{2+} concentration in porcine cultured aortic vascular smooth muscle cells (Feth *et al*., 1993). These data support the view that the inhibitory effects of pp56 occur distal to NPY receptors and indicate that the most commonly identified signal transduction mechanisms are not involved. Alternatively, the inhibitory action of pp56 may be associated with receptor subtypes utilizing alternative signalling mechanisms, such as the regulation of phosphatidylinositol metabolism by PLCβ.

The ability of pp56 to interact selectively with PLCβ-mediated contraction in cardiomyocytes is established by the evidence presented here that the response to noradrenaline, used as a positive control, was abolished completely, whereas responses to isoprenaline and forskolin, which both act through adenylate cyclase and were included therefore as negative controls, were not affected. It had been suggested previously that PLCβ-dependent mechanisms are associated with the actions in cardiomyocytes of NPY, as shown by its attenuation of basal phosphatidylinositol turnover (Xiang & Brown, 1993), of endothelin-1, as indicated by stimulation of inositol 1,4,5 trisphosphate production (de Jonge *et al*., 1995) and of CGRP, as inferred by its ability to activate protein kinase C (Bell *et al*., 1995). In contrast, the effects of secretin and VIP in these cells are not PLCβ-dependent, the receptors being coupled to the accumulation of cyclic AMP (Bell & McDermott, 1994a). The findings of the present study that responses to NPY, endothelin-1 and CGRP are inhibited by pp56, whereas those to VIP and secretin are not, substantiate the previous indications about signal transduction mechanisms and add further weight to the suggestion that pp56 might distinguish between receptors coupled to the regulation of phosphatidylinositol metabolism and those coupled to phosphatidylinositol-independent signal transduction pathways. It is of interest to note here that receptors for ATP and thrombin can also couple to the activation of PLCβ (reviewed in de Jonge *et al*., 1995), since the effects of these stimuli can also be attenuated by pp56 in a range of vascular tissues (Winter *et al*., 1989; Sun *et al*., 1991; 1992; Wahlestedt *et al*., 1992).

The molecular structure of pp56 indicates that an interaction with an inositol 1,4,5 trisphosphate-dependent mechanism and/or receptor would be the most probable mechanism of action. However, this is unlikely for two reasons. Most importantly, the hydrophillic nature of pp56 does not accord with the theory that the compound crosses the cell membrane to act on inositol 1,4,5 trisphosphate-dependent processes or other intracellular targets, although such diffusion barriers would be much less in isolated cells than in multicellular tissues *in vivo* or *in vitro*. Also, the binding data obtained in a number of tissues, including partially purified myocardial membranes, have indicated that [^{3}H]-pp56 membrane binding sites are more similar to [^{3}H]-inositol 1,3,4,5 tetrakisphosphate binding sites in terms of their distribution and pH and Ca^{2+} dependencies than to [^{3}H]-inositol 1,4,5 trisphosphate binding sites (Yoo *et al*., 1994; Walsh *et al*., 1995). In some tissues, inositol 1,3,4,5 tetrakisphosphate interacts with specific cell surface receptors to regulate the influx of Ca^{2+} and replenish the inositol 1,4,5 trisphosphate-sensitive intracellular pools of Ca^{2+}. Although inositol 1,3,4,5 tetrakisphosphate binding sites are present in myocardial tissues, the ability of such receptors to modulate the influx of Ca^{2+} across the sarcolemma and thereby regulate contractility in cardiomyocytes has not been established (reviewed in de Jonge *et al*., 1995). However, such a mechanism would offer a conceivable explanation for the ability of pp56 to inhibit selectively inositol phosphate-dependent contractile-coupling processes without crossing the hydrophobic sarcolemma. A possible model for the action of pp56 in the cardiac myocyte is that of use-dependent blockade of inositol 1,3,4,5 tetrakisphosphate-regulated calcium channels located on the sarcolemma. It is envisaged that agonist-stimulated accumulation of inositol 1,3,4,5 tetrakisphosphate intracellularly would increase the proportion of inositol 1,3,4,5 tetrakisphosphate-regulated channels that are in the open (activated) configuration; opening of channels would expose their inner protein lining, in which common inositol 1,3,4,5 tetrakisphosphate/pp56 binding sites are embedded, to the action of pp56. This model of 'use-dependent' inhibition is supported by the observation in this study of the marked temporal dependence of the inhibition by pp56 of the effect of noradrenaline, contrasted with the lack of significant effect of pp56 with time on the basal contractile amplitude, under which conditions few channels would be in the open state.

In conclusion, these data call into question the previously demonstrated selectivity of pp56 as an antagonist of NPY-mediated cellular processes and raise the possibility that, in ventricular cardiomyocytes, pp56 appears to be a useful pharmacological agent with which to distinguish between PLC-dependent and PLC-independent contractile coupling processes. However, the exact mechanism by which pp56 exerts its inhibitory effects has not been determined and may involve a specific interaction with a part of the inositol phosphate signalling cascade other than the inositol 1,4,5 trisphosphate receptor present on sarcoplasmic reticulum membranes.

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