SRIF Receptor Subtype Expression and Involvement in Positive and Negative Contractile Effects of Somatostatin-14 (SRIF-14) in Ventricular Cardiomyocytes

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Key Words

Receptor expression • Heart • Rat • Inotropic • Ventricle • Somatostatin agonists • L808087 • L796778

Abstract

Background/Aims: Somatostatin-14 (SRIF-14), a neuropeptide co-stored with acetylcholine in the cardiac parasympathetic innervation, exerts both positive and negative influences directly on contraction of ventricular cardiomyocytes, indicative of involvement of more than one of five known SRIF (SSTR) receptor subtypes. The aim was to characterize receptor subtype expression in adult rat ventricular cardiomyocytes and to investigate the influence of a series of SRIF (SSTR) subtype-selective agonists on contractile parameters. **Methods:** mRNA and protein expression of each receptor subtype were quantified by RT-PCR and immunoblotting respectively; for contraction studies, cells were stimulated at 0.5 Hz under basal conditions and in the presence of isoprenaline (ISO, 10^{-8} M). **Results:** all five SRIF (SSTR) receptor subtypes were expressed in cardiomyocytes although SRIF1A (SSTR2) and SRIF2A (SSTR1) were less abundant than the other subtypes. L803087 (10^{-8} M), a SRIF2B

(SSTR4) agonist, attenuated ISO-stimulated peak contractile amplitude and prolonged relaxation time (T_{50}). L796778 (10^{-7} M), a SRIF1C (SSTR3) agonist, augmented basal and ISO-stimulated peak contractile amplitude; L779976 (10^{-8} M) and L817818 (10^{-9} M), agonists at SRIF1A (SSTR2) and SRIF1B (SSTR5) receptors, respectively, also augmented ISO-stimulated peak amplitude. **Conclusion:** these data support involvement of SRIF2B (SSTR4) receptors in the negative contractile effects of SRIF-14, while one or more of the three SRIF1 receptor subtypes (SSTR2, 3 or 5) may contribute to the positive contractile effects of SRIF-14.

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Introduction

Somatostatin-14 (SRIF-14) is a tetradecapeptide [1] which was identified initially in the hypothalami of sheep [2]. SRIF-14 is synthesised by post-translational cleavage of the precursor pro-peptide, somatostatin-28 (SRIF-28) [3]. SRIF-like immunoreactivity is co-localised with acetylcholine in the local postganglionic parasympathetic innervation supplying the heart [4-6].

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SRIF-14 exerts a negative inotropic effect in adrenaline-stimulated canine Purkinje fibres by a mechanism which may be attributed, at least in part, to a reduction in transmembrane calcium influx [7]. In addition, the peptide inhibits the positive inotropic response to isoprenaline in papillary muscles of guinea pigs without altering basal contractile tension [8]. Data obtained previously in our laboratory [9] indicate that SRIF-14 exerts dual contractile effects directly on rat ventricular cardiomyocytes: (1) a positive contractile effect, associated with activation of an L-type calcium channel current; (2) a negative contractile effect, more pronounced in the presence of the β -adrenoceptor agonist, isoprenaline, and attributed at least in part to activation of a transient outward potassium current, I_{to} . The dual nature of the influence of the peptide supports the probable involvement of multiple receptor subtypes in the regulation of cardiomyocyte contractility by SRIF-14.

Cloning and expression of SRIF receptor proteins (also known as somatostatin transfected receptors, SSTRs) has indicated the existence of at least 5 receptor subtypes, divided into two groups, distinguishable on the basis of their affinity for octreotide and seglitide [10, 11]. SRIF1 receptors, which possess high affinity for these SRIF analogues, have been subdivided into 3 receptor subtypes, SRIF1A (SSTR2), SRIF1B (SSTR5) and SRIF1C (SSTR3), while SRIF2 receptors, which possess lower affinity for these analogues, have been subdivided into 2 subtypes, namely SRIF2A (SSTR1) and SRIF2B (SSTR4) [11, 12]. Octreotide has been found to ameliorate left ventricular hypertrophy and improve cardiac function in patients with acromegaly [13]; it is unclear whether the beneficial effects of this analogue are due indirectly to attenuation of Growth Hormone and IGF-1 secretion [14] or whether SRIF receptor agonists exert additional effects directly on the heart.

All 5 receptor subtypes belong to the G-protein coupled receptor superfamily [15] and are primarily coupled with a variety of pertussis toxin-sensitive G-proteins, including $G_{i\alpha1}$, $G_{i\alpha3}$, $G_{o\alpha}$, $G_{\beta36}$ and $G_{\gamma3}$ [16] thereby supporting a wide range of cell signaling events, such as inhibition of adenylate cyclase [17] and L-type calcium channels [18] and activation of potassium channels [19], underpinning the diverse functional effects of the peptide. Furthermore, at least in the case of the three SRIF1 receptor subtypes (SSTR2, 3 and 5), coupling to the phospholipase C- β signaling cascade via pertussis toxin-insensitive $G_{aq/11}$ G-protein has also been proposed [20]. While physiological roles have been identified for the other SRIF (SSTR) receptor subtypes [21-25], the

function of SRIF2B (SSTR4) receptors is less established; putative functions include involvement in regulation of memory [26], inhibition of sensory neuropeptide release [27] and inhibition of neointimal hyperplasia [28].

The few reports to date of cardiac expression of the various SRIF (SSTR) receptors have indicated variation between species, developmental stage and cellular type. SRIF2B (SSTR4) is the major isoform expressed at mRNA level in the adult rat heart [29] with lesser amounts of SRIF1C (SSTR3) and SRIF2A (SSTR1) and the absence of SRIF1A (SSTR2) and SRIF1B (SSTR5) mRNAs; in contrast SRIF2B (SSTR4) mRNA is not expressed in the adult mouse heart [30]. The embryonic rat cardiomyocyte cell line H9c2 expresses all five SRIF (SSTR) receptors [31], while expression of SRIF1B (SSTR5) mRNA can be induced in neonatal cardiomyocytes in response to the cytokine, leukemia inhibitory factor [32]. Smith and coworkers [33] have recently reported the presence of SRIF1A (SSTR2), SRIF1B (SSTR5), SRIF2A (SSTR1) and SRIF2B (SSTR4), but not SRIF1C (SSTR3) receptor mRNA in both atrial and ventricular tissues from human heart; while cardiac fibroblasts expressed all four of these receptors, cardiomyocyte expression was restricted to SRIF1A (SSTR2) and SRIF2A (SSTR1).

Lack of highly selective agonists and antagonists for each of the five receptor subtypes has until relatively recently hindered functional studies to characterize receptor-subtype involvement in the various physiological actions of the peptide. The development of a series of high affinity, subtype selective non-peptide agonists for each of the SRIF receptors has greatly facilitated definition of their respective physiological functions [34]. The objective of the study therefore was to characterize SRIF (SSTR) receptor subtype expression in adult rat ventricular cardiomyocytes and to examine receptor subtype involvement in direct regulation of cardiomyocyte contractile and hypertrophic parameters in vitro utilizing the series of non-peptide subtype-selective agonists developed by Merck Research Laboratories.

Materials and Methods

Experimental model

The study was performed in accordance with Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*, published by Her Majesty's Stationary Office, London. Male Sprague-Dawley rats were obtained at 12 weeks of age from a colony maintained at the QUB Laboratory Service Unit.

Table 1. Primers Sequences. Accession Numbers are taken from the European Molecular Biology Laboratory (EMBL) database which is part of the International Nucleotide Sequence Database Collaboration.

	Forward	Backward
SRIF 2A (SSTR1) X62314	CAG GGT AGC GCC ATT CTC A 19bp [307-325]	TGT TGG TTG CGG TCT TCA TC 20bp [408-427]
SRIF 1A (SSTR2) M93273	CCCCTATGTAATCTCGT 18bp [1800-1817]	TGCAAGAGGGATGCTG 16bp [2009-2024]
SRIF 1C (SSTR3) X63574	TAAGGCACGAGTACCAGACAC 21 bp [1996-2016]	ACTTGGGCTCTGATTAGGAG 20bp [2275-2294]
SRIF 2B (SSTR4) M96544	TGTCCCTCATCTCAGCTATG 21 bp [1082-1102]	CTCAGCACCTCCAGTTGTTTC 21 bp [1198-1218]
SRIF 1B (SSTR5) L04535	CGCCAAGATGAAGACAGTTAC 21 bp [348-368]	TGACCATCAGGCAGAAGATAC 21bp [524-544]
GAPDH AB017801	GAAACCCATCACCATCTTCCA 21bp [248-268]	ACCCCATTTGATGTTAGCGG 20bp [280-299]

Cardiomyocyte isolation

Following deep anesthesia of the rats using isoflurane (Abbott Laboratories, UK), the hearts were rapidly excised and placed in ice-cold saline. Excised hearts were cannulated through the ascending aorta, and preparations of ventricular cardiomyocytes were isolated by enzymatic digestion (collagenase, 0.4mg/ml, Serva, Germany) using Langendorff perfusion [35]. After purification, cells were utilized immediately for extraction of RNA; for analysis of contractile and hypertrophic parameters, the cell preparation was suspended at a concentration of approximately 80,000 viable cardiomyocytes/ml in a 'creatinine-carnitine-aurine' (CCT) medium which consisted of modified glutamine-free medium M-199 supplemented with Earle's salts (Gibco, UK), HEPES (15 mM), creatinine (5 mM), *L*-carnitine (2 mM), taurine (5 mM), ascorbic acid (100 µM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Contractile experiments were conducted in parallel with hypertrophic and expressional studies also requiring routine isolation of cardiomyocytes from healthy control Sprague-Dawley rats at 12 weeks of age in order to minimize the total number of animals required for the study.

Real-Time PCR

Total cellular RNA was isolated using acid guanidinium thiocyanate-phenol-chloroform extraction and alterations in mRNA expression determined by RT-PCR. Reported sequences for each gene (Table 1) were used to design on Primer Express software (PE Applied Biosystems), rat specific primers adapted to RT-PCR conditions, which were synthesized by Invitrogen. RT-PCR was performed using the following cycle parameters: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C using the ABI Prism Sequence Detector (PE Applied Biosystems). For each gene, RT-PCR was conducted in duplicate using ABsolute QPCR Sybr Green ROX (Abgene) in a 2:1 reaction. To ensure the quality of measurements, both negative and positive controls were included in each plate.

The threshold cycle (Ct) value, at which a statistically significant increase in signal, associated with an exponential growth of PCR product was observed, was used to ascertain expression level. Analysis was performed using ABI 7000 Prism software and statistical analysis of the RT-PCR results were performed using the Ct value (Ct gene of interest -Ct reporter gene). Relative gene expression was obtained by Ct methods (Ct sample -Ct calibrator) using the control group as a calibrator for comparison of every unknown sample gene expression level. The conversion between Ct and relative gene expression level is n fold induction = $2^{-\Delta\Delta Ct}$. Comparisons between genes are based on the assumption that PCR efficiency is similar for each cDNA amplified; it is acknowledged that if this assumption is invalid, some bias might be introduced into the analysis since each cDNA is amplified using a distinct set of primers.

Preparation of membrane protein

Viable cardiomyocytes were suspended in a HEPES (20 mmol/L)-based homogenisation buffer (pH 7.4) containing protease inhibitors (Sigma Chemical Company, UK): aprotinin (0.8 µmol/L), bacitracin (0.1 mmol/L), benzamidazine (0.1 mmol/L), EDTA (5 mmol/L), leupeptin (2 µmol/L), PMSF (0.1 mmol/L) and sonicated on ice for 3 bursts of 10 sec at 25 watts (VIBRA-CELL sonicator, Sonics & Materials Inc., Danbury, CT) and then centrifuged at 4°C for 10 min at 1000 r.p.m. (Sigma 3K18) to sediment cell nuclei and mitochondrial fractions. The resulting supernatant, containing a crude fraction of plasma membranes, was centrifuged at 12,000 r.p.m. for 55 min at 4°C and the pellet formed washed with homogenisation buffer supplemented with detergent, n-Octylglucoside (0.06mM), sonicated for 3 bursts of 10 sec at 25 Watts and incubated on ice for a further period of 15 min. Finally, the suspension was centrifuged at 12,000 r.p.m. for 55 min at 4°C and the pellet formed washed with homogenisation buffer containing detergent and sonicated for 20 sec at 25 Watts prior to storage at -70°C pending analysis.

Fig. 1. SRIF (SSTR) receptor subtype mRNA expression in adult rat ventricular cardiomyocytes (a). Data are normalized to GAPDH mRNA levels and expressed as $2^{-\Delta\Delta Ct}$ (b) and are the mean values + SE (n=10 cardiomyocyte preparations). *variation from SRIF1A (SSTR2) levels (*0.01<P<0.05).

Immunodetection and Quantification

Membrane protein concentration was determined by the method of Lowry. Protein samples were mixed with 4μl of 1:1 mercaptoethanol: loading buffer (0.5M Tris 25% v/v, glycerol 20%v/v, 10% sds 40% v/v, H₂O 15% v/v, Bromophenol blue 0.005% w/v) prior to separation by 12% SDS-PAGE (20-80μg protein per lane) and transfer to PVDF membrane (0.45μm, Millipore, UK). The PVDF membrane was washed with phosphate buffered saline (PBS) containing 0.1% v/v Tween 20 (Sigma, UK) and blocked overnight in PBS/0.1% v/v Tween 20 solution containing 5% w/v Marvel. Immunoblotting was performed using primary antibodies directed specifically against rodent SSTRs (SRIF receptors) (Santa Cruz Biotechnology, sc-11614, sc-11620, sc-11624 raised in goat, sc-25675, sc-25676 raised in rabbit) used at a dilution of 1:500. Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (donkey anti-goat sc-2020 or donkey anti-rabbit sc-2313 used at a dilution of 1:20000, Santa-Cruz Biotechnology) and ECL plus (Amersham Biosciences, UK) as substrate, and quantified by densitometry (Analytical Imaging System) normalized for protein loading using β-actin (Santa Cruz Biotechnology, sc-1616 raised in goat).

Contractile parameters

An aliquot of cardiomyocyte suspension (150 μl) was placed in a transparent recording chamber mounted on an inverted phase-contrast microscope (Axiovert IM35, Carl Zeiss, Germany), and allowed to settle for 10 min before being bathed with oxygenated Krebs-Henseleit buffer. The composition of the Krebs-Henseleit solution was (in mmol/L): NaCl (120); KCl (4.7); NaHCO₃ (25); MgSO₄ (0.97); KH₂PO₄ (1.2); glucose (11); Ca²⁺ (1 or 3); prior to use, this solution was gassed with 95% O₂ / 5% CO₂ for 20 minutes and maintained at pH 7.4.

Cells were field-stimulated at 0.5Hz with biphasic pulses of 0.5ms duration at 60 V via Ag/AgCl wires embedded in the wall of the chamber. Contractile parameters were determined at baseline and after incubation for 5 minutes with a sub-maximal [36] concentration of isoprenaline (ISO, 10⁻⁸M). Following washout, cells were incubated for a further 5 minutes with SRIF-14 (Bachem, UK) or receptor subtype-selective agonist (Merck Research Laboratories, New Jersey, USA) and contractile parameters again assessed. Finally, cells were incubated with isoprenaline (ISO, 10⁻⁸M) in the presence of SRIF-14 or receptor agonist for a further 5 minutes after which contractile parameters were again assessed. The video-edge detection equipment and the procedure used in the measurement were described previously [36, 37]: baseline peak amplitude does not differ significantly within the time interval of this experimental protocol (<1 hour), although basal peak amplitude and isoprenaline response both decline (p<0.005) following washout and repeated challenge. However, when adjusted for reduction in baseline, a subsequent isoprenaline challenge yields an identical response to that of the first challenge. The entire experimental protocol was performed only once in each cell i.e. the response to only one SRIF agonist was studied in each cell. On a typical day, satisfactory results were obtained from 2-4 ventricular cardiomyocytes from each heart cell preparation due to the constraints of the length of the experimental protocol.

Hypertrophic parameters

Petri dishes (35mm diameter) were pre-incubated for 2 hours with fetal calf serum (4% v/v) in CCT Medium. Aliquots of cell suspension (1 ml) were pipetted gently onto Petri dishes, and after 1 h viable cardiomyocytes became attached to the surface of the dish. The dishes were then washed with fresh

Fig. 2. SSTR (SRIF receptor subtype) protein expression in adult rat ventricular cardiomyocytes. Representative immunoblots depicting the molecular weight of each SSTR (a); data are expressed relative to β -actin levels (b) and are the mean values + SE ($n=4-8$ cardiomyocyte preparations). *variation from SRIF1A (SSTR2) levels (* $0.01 < P < 0.05$; *** $P < 0.005$).

CCT medium to remove non-attached cells and cell debris and the attached cells were exposed for 24 h to L-U- ^{14}C phenylalanine (0.1 $\mu\text{Ci}/\text{ml}$ culture medium; Amersham Pharmacia Biotech, UK). Incorporation of radioactivity into the acid-insoluble cell fraction was determined under basal conditions and in the presence of a hypertrophic stimulus, the protein kinase C activator, phorbol 12 myristate 13 acetate (PMA, 10^{-7} M) $\pm$ SRIF receptor agonist at an optimally selective concentration for each agonist [34]. The attached cells were then washed with an aliquot (1 ml) of ice-cold PBS, prior to the addition of an aliquot (1 ml) of ice-cold trichloroacetic acid (10% w/v). After storage overnight at 4°C , the acid containing the intracellular precursor pool was removed from the dishes and the attached cells were washed with an aliquot (1 ml) of PBS. The precipitate remaining on the culture dishes was dissolved in an aliquot (1 ml) of NaOH (0.1 M)/sodium dodecyl sulphate (0.01% w/v) by overnight incubation at 37°C . In these samples, concentration of DNA was determined by a spectrophotometric method in which bisbenzamide dye was incorporated into DNA, protein was assessed by the colorimetric method of Lowry, and the radioactivity was counted [35]. The ratios of L-U- ^{14}C phenylalanine incorporated to DNA per culture and protein mass to DNA per culture served as measures of *de novo* synthesis of cellular protein and of cellular protein mass, respectively.

Data analysis

Data are expressed as means $\pm$ SE where n denotes number of heart cell preparations used to analyze mRNA expression or hypertrophic parameters or number of cells used to measure contractile amplitude. Contractile amplitude was measured in μm and expressed as a percentage change from the diastolic length ($\delta\text{L}\%$). Statistical analyses were performed by analysis of variance to detect significant differences and post-hoc comparisons by Student's paired t test. Values of $P < 0.05$ were considered significant.

Results

Receptor subtype expression

SRIF1B (SSTR5), SRIF1C (SSTR3) and SRIF2B (SSTR4) receptor mRNAs were expressed abundantly in ventricular cardiomyocytes while SRIF1A (SSTR2) and SRIF2A (SSTR1) receptor mRNAs were detected only at low levels (Fig 1). Similarly, SSTR5 (SRIF1B), SSTR3 (SRIF1C) and SSTR4 (SRIF2B) receptor proteins were abundant on membranes from ventricular cardiomyocytes, represented by bands of molecular weights approximately 52, 80 and 45 kDa, respectively; SSTR2 (SRIF1A) and SSTR1 (SRIF2A) receptors, represented by bands of 52 kDa and 42 kDa, respectively, were found at lower levels (Fig 2).

Contractile parameters

As indicated in Fig. 3, isoprenaline (ISO, 10^{-8} M) increased the mean extent of contractile shortening (by 41% of basal response, $P < 0.005$), yet reduced mean time taken to reach peak amplitude of shortening (by 11% of basal response, $P < 0.01$), indicative of increased shortening velocity. Isoprenaline initially delayed the mean relaxation time (T_{50} increased by 9% of basal response, $P < 0.05$), although taking account of increased peak shortening, the mean velocity of relaxation was actually enhanced. SRIF-14 (10^{-8} M) did not influence the mean values of any of the contractile parameters significantly either under basal conditions or in the presence of isoprenaline. However, the cells in which the response to SRIF-14 was studied ($n=18$) could be divided into two groups based on a positive

Fig. 3. Effect of isoprenaline (10^{-8} M) on peak contractile amplitude (a); time to peak amplitude (b) and time to 50% relaxation, T_{50} (c) in ventricular cardiomyocytes, elicited in the absence and in the presence of SRIF-14 (10^{-8} M). Peak change in cell amplitude is expressed as a percentage ($\delta L\%$) of resting cell length (L). Data are mean values $\pm$ SE ($n=18$ cells). *variation between baseline and isoprenaline response ($0.01 < P < 0.05$; ** $0.005 < P < 0.001$; *** $P < 0.005$). + variation between baseline responses before and after washout ($+ 0.01 < P < 0.05$; +++ $P < 0.005$); influence of SRIF-14 (10^{-8} M) on peak contractile amplitude under basal and isoprenaline-stimulated conditions (d): composite mean of all cells ($n=18$), cells evoking a positive response ($n=12$) and cells evoking a negative response ($n=6$); difference from respective basal or isoprenaline-stimulated response ($0.01 < P < 0.05$; ** $0.005 < P < 0.001$; *** $P < 0.005$).

($n=12$) or negative ($n=6$) influence of SRIF-14 on basal amplitude and on isoprenaline-stimulated peak amplitude of each cell (Fig 3d).

The effects of each of the SRIF receptor agonists on basal and isoprenaline-stimulated peak amplitude are summarized in Fig.4a. In each case, the concentration of agonist employed was chosen to maximize the ability to

discriminate between the various SRIF receptor subpopulations based on published data (Rohrer et al., 2000, Table III). SRIF-14 responses are included for comparison. SRIF1C (SSTR3) agonist, L796778 (10^{-7} M) consistently increased basal and isoprenaline-stimulated peak amplitude ($P < 0.05$) in all cells tested. SRIF1A (SSTR2) agonist, L779976 (10^{-8} M), and SRIF1B (SSTR5)

Fig. 4. Influence of SRIF (SSTR) receptor agonists on (a) peak contractile amplitude; (b) time to peak amplitude; (c) time to 50 % relaxation elicited under basal conditions (i) and in the presence of isoprenaline (ISO, 10^{-6} M) (ii) in ventricular cardiomyocytes. Peak change in contractile amplitude is expressed as a percentage ($\delta L\%$) of resting cell length (L). Data are mean values $\pm$ SE (SRIF-14 $n=18$ cells; SRIF agonists $n=3-6$ cells). *variation between responses elicited in the absence and in the presence of SRIF agonist ($*0.01 < P < 0.05$).

agonist, L817818 (10^{-9} M), also augmented isoprenaline-stimulated peak amplitude consistently in all cells tested.

In contrast, of all the compounds tested only the SRIF2B (SSTR4) agonist, L803087 (10^{-8} M), consistently

Fig. 5. Effect of SRIF-14 and SRIF receptor agonists on (a, c) protein synthesis; (b, d) protein mass in ventricular cardiomyocytes (combined population obtained from left and right ventricles of 12 week old normotensive control rats) and maintained in short-term (24 h) culture under basal conditions (a, b) and in the presence of the hypertrophic stimulus, PMA (10^{-7} M) (c, d). Data are the mean values + SE of 4 heart cell preparations. *denotes significant difference between basal value and PMA-stimulated response ($P < 0.05$).

attenuated isoprenaline-stimulated peak amplitude while SRIF2A (SSTR1) agonist, L797591 (10^{-8} M) had no discernable effect.

The effects on rise time are shown in Fig. 4b. None of the agonists tested influenced basal rise time. L803087 attenuated while L817818 augmented isoprenaline-stimulated rise time consistently, reflecting in part the influences of these agonists on the magnitude of the peak shortening amplitude. The effects on relaxation time (T_{50}) are shown in Fig. 4c. None of the agonists tested

influenced basal T_{50} . L803087 prolonged T_{50} consistently in isoprenaline-stimulated cells ($P < 0.05$), reflecting a slower rate of relaxation despite reduced peak amplitude; L817818 tended to prolong T_{50} in isoprenaline-stimulated cells which could largely be accounted for by augmented peak amplitude in the presence of this agonist.

Hypertrophic parameters

The phorbol ester PMA (10^{-7} M) employed as a positive control for hypertrophic growth, increased

cardiomyocyte protein synthesis (Fig. 5a, c) and cellular protein mass ($P < 0.05$) (Fig. 5b, d). Neither SRIF-14 nor any of the SRIF receptor-subtype selective agonists, applied at concentrations to maximize the ability to discriminate between the various SRIF receptor subpopulations, attenuated baseline (Fig. 5a, b) or PMA-stimulated (Fig. 5c, d) protein synthesis and protein mass.

Discussion

SRIF1B (SSTR5), SRIF1C (SSTR3) and SRIF2B (SSTR4) receptors were expressed abundantly in adult rat ventricular cardiomyocytes; SRIF1A (SSTR2) and SRIF2A (SSTR1) receptors were expressed less abundantly. It is not uncommon for individual tissues or cell types to co-express more than one SRIF (SSTR) receptor subtype. Indeed, Granata and coworkers [31] have detected all five SRIF (SSTR) receptor subtypes in the embryonic rat cardiomyocyte cell line H9c2; however, it is not clear how expression levels in this immature cell line relate to that of adult myocardium. Although they did not quantify the levels of expression of each receptor, the immunoblots depicted in their study indicate that SSTR5 (SRIF1B) and SSTR3 (SRIF1C) were the most abundantly expressed receptors, in agreement with data obtained in our laboratory. In agreement with the present study employing RT-PCR, Bruno and co-workers [29] detected SRIF1C (SSTR3), SRIF2A (SSTR1) and SRIF2B (SSTR4) mRNAs, but in contrast to our observations, were unable to detect expression of SRIF1A (SSTR2) or SRIF1B (SSTR5) mRNAs in adult rat heart possibly reflecting the lesser sensitivity of the nuclease protection methodology then available. It should also be noted that Bruno's group extracted RNA from a crude preparation comprising multiple cardiac cell types which might vary in regard to their respective contribution to overall expression levels of each receptor detected within the tissue. Using a similar approach, other investigators have been unable to detect SRIF1B (SSTR5) [38] or SRIF2B (SSTR4) [30] receptor expression in adult murine heart. In a study by Smith and co-workers [33], human cardiomyocytes were found to express only SRIF1A (SSTR2) and SRIF2A (SSTR1) receptors, in contrast to human cardiac fibroblasts, which in addition to these subtypes, expressed SRIF1B (SSTR5) and SRIF2B (SSTR4) but not SRIF1C (SSTR3) receptors. Rodent and human myocardium clearly differ in regard to SRIF receptor subtype expression, since we have found that SRIF1A (SSTR2) and SRIF2A (SSTR1) are the least

abundant receptors expressed in adult rat ventricular cardiomyocytes. Interestingly, we have also detected SRIF1B (SSTR5), SRIF2A (SSTR1) and SRIF2B (SSTR4) mRNAs in a crude preparation of adult rat cardiac non-myocytes, representing predominantly cardiac fibroblasts, albeit at significantly lower expression levels than in cardiomyocytes (McMaster and Zhao, unpublished observation). Overall, a common finding in all published studies in regard to the heart, and cardiomyocytes specifically, would appear to be that at least two receptor subtypes, typically one of the SRIF1 subtype (SSTR2, 3 or 5) and 1 of the SRIF2 subtype (SSTR 1 or 4), are expressed. This observation is compatible with our previous finding that SRIF-14 exerts a dual influence on ventricular cardiomyocyte contractility, implying the expression of multiple receptor subtypes in this cell population [9]. This is consistent with the finding that the mean contraction response of the group of cardiomyocytes employed in the present study was not altered significantly by SRIF-14 itself but was influenced by subtype-selective agonists. Furthermore, all of the cells studied could be divided into two groups on the basis of whether the overall response to SRIF-14 was positive or negative in each cell which would in turn depend on the relative abundance of expression of each receptor subpopulation on an individual cell.

Of all the non-peptide SRIF (SSTR) receptor agonists tested, only L803087 attenuated isoprenaline-stimulated peak contractile amplitude. At the concentration employed (10^{-8} M) this compound displays high selectivity for SRIF2B (SSTR4) receptors; interaction with other receptors would be expected to be negligible since L803087 binds to the three SRIF1 receptor subtypes with only micromolar affinity [34]. The absence of any discernable contractile effect of L797591 (10^{-8} M), together with the low level of expression of SRIF2A (SSTR1) receptor, indicates that cross-reaction of L803087 at this receptor can be discounted. These data indicate that the negative inotropic effect of SRIF-14 can be attributed to activation of the SRIF2B (SSTR4) receptor subtype. Activation of the transient outward potassium current, I_{to} , accounts for the majority of the negative inotropic effect of SRIF-14 in isoprenaline-stimulated cardiomyocytes [9] but inhibition of adenylate cyclase resulting in attenuated production of the second messenger substance, cyclic AMP, may also make a contribution [8]. Activation of I_{to} and decreased production of cyclic AMP both influence the activity of L-type calcium channels resulting in reduced influx of calcium ion and opposing isoprenaline-induced increase in shortening velocity and

peak amplitude. Rat SRIF2B (SSTR4) receptors are known to couple to inhibition of adenylate cyclase and activation of potassium channels via inhibitory G-proteins [19, 39, 40].

L796778, a partial agonist at SRIF1C (SSTR3) receptors [34], augmented peak amplitude under basal conditions and in isoprenaline-stimulated cells. This compound is devoid of activity at SRIF1A (SSTR2) receptors and at the concentration chosen (10^{-7} M) has negligible activity at the other SRIF (SSTR) receptor subtypes. The marked effect of this partial agonist strongly supports a role for the SRIF1C (SSTR3) receptor in mediating the positive contractile effects of SRIF-14; indeed the suboptimal efficacy of 60% underestimates the true magnitude of response that could be generated by a full agonist acting at this receptor.

L779976 and L817818 also augmented isoprenaline-stimulated peak amplitude consistently in all cells tested. Interaction of L779976, a SRIF1A (SSTR2) subtype-selective agonist, with other SRIF receptor subtypes would be expected to be minimal at the concentration employed in the present study (10^{-8} M); limited cross-reactivity at other SRIF receptors, most notably SRIF2B (SSTR4), is only observed at much higher concentrations [34]. Furthermore all of the cardiomyocytes tested with this compound exhibited a positive response in contrast to the negative response consistently observed in response to the SRIF2B (SSTR4) agonist, L803087. Despite the limited expression of the SRIF1A (SSTR2) receptor in cardiomyocytes, a contribution of this subtype to the positive contractile effect of SRIF-14 cannot be discounted particularly in view of the relatively high affinity of the natural ligand for this receptor subtype [34]. Of all of the agonists tested, L817818 displays the least selectivity; although preferentially binding to SRIF1B (SSTR5) receptors significant interaction is also observed at SRIF2A (SSTR1) receptors, and to a considerably lesser extent at the remaining SRIF receptor subtypes. The absence of effect of L797591, coupled with the low levels of expression of SRIF2A (SSTR1) receptors, indicate that an interaction with this subtype can be discounted. The concentration of L817818 at which augmentation of peak amplitude was observed (10^{-9} M) was also 50-100 fold less than the reported K_i values for cross-reactivity of binding at SRIF1A (SSTR2), SRIF1C (SSTR3) and SRIF2B (SSTR4) receptors [34] indicating that SRIF1B (SSTR5) receptors, expressed abundantly in cardiomyocytes, could make an independent contribution to the positive contractile effect of SRIF-14.

The signaling cascade associated with the positive contractile effect of SRIF-14 is likely to involve stimulation of the phospholipase C pathway, mobilization of intracellular calcium and L-type calcium channel activation [9] thereby affording opportunity for synergy with isoprenaline-mediated stimulation of β -adrenoceptors coupled to activation of the cyclic AMP pathway. All three SRIF1 receptor subtypes are known to activate phospholipase C- β by a Gq dependent mechanism [17, 20, 41].

SRIF-14 has been shown to inhibit cell growth and proliferation, including growth of endothelial cells and angiogenesis; these effects have been attributed to activation of protein tyrosine phosphatases and modulation of the MAPK signaling cascade [22, 42]. However, neither SRIF-14 nor any of the receptor subtype-selective agonists attenuated cardiomyocyte hypertrophy directly, as evidenced by the lack of effect on cellular protein synthesis and mass, under basal conditions or in the presence of a hypertrophic stimulus, PMA. In contrast to SRIF-14, another cardioendocrine peptide, adrenomedullin, did attenuate PMA-stimulated cardiomyocyte protein synthesis under identical experimental conditions to those employed in the present investigation [35]. This finding supports the hypothesis that regression of left ventricular hypertrophy in acromegalic patients in response to octreotide therapy is mediated indirectly through reduction in circulating growth hormone and insulin-like growth factor 1 levels [13, 14] since SRIF-14 inhibits growth hormone secretion from the anterior pituitary [43] and growth hormone is a known stimulus for secretion of the insulin-like growth factors [44]. Furthermore, data obtained previously in our laboratory indicate that IGF-1 exerts a hypertrophic action directly in adult cardiomyocytes [45]. Left ventricular hypertrophy in acromegaly is characterized not only by hypertrophy of cardiomyocytes, which are terminally differentiated and unable to proliferate, but also disproportionate proliferation of cardiac fibroblasts and accumulation of fibrillar collagen contributing to impaired diastolic function [13, 14]. In view of the presence of SRIF (SSTR) receptors on human cardiac fibroblasts [33] a direct inhibitory influence of SRIF-14 analogues on fibroblast proliferation cannot be discounted at present.

In conclusion, all 5 SRIF receptor subtypes are expressed in adult rat ventricular cardiomyocytes, with SRIF1B (SSTR5), SRIF1C (SSTR3) and SRIF2B (SSTR4) expressed more abundantly than SRIF1A (SSTR2) and SRIF2A (SSTR1). The negative contractile effect of SRIF-14 is most probably associated with

activation of the SRIF2B (SSTR4) subtype while activation of SRIF1C (SSTR3) receptors is associated with the positive contractile effect of this peptide. An additional contribution of the other SRIF 1 receptor subtypes to the latter is proposed; indeed functional interactions between different SRIF receptor subtypes co-expressed within the same cell are not uncommon and can afford greater flexibility of response [46].

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