SUPPLEMENTAL DATA

Journal of Pharmacology and Experimental Therapeutics

Manuscript number: JPET-AR-2022-001335

"Istaroxime metabolite PST3093 selectively stimulates SERCA2a and reverses disease-induced changes in cardiac function" by Arici M & Ferrandi M et al.

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Supplementary Methods

Animal models

Male Sprague Dawley (SD) rats (150-175 gr) were used to generate STZ-induced diabetic cardiomyopathy model to test compounds *in vivo* and *in vitro*; female Dunkin-Hartley guinea pigs (175-200 g) were used for cardiac and skeletal muscle preparations (male 450-500 g) and myocytes isolation and finally, male Albino Swiss CD1 mice (30 g) were used for acute *in vivo* toxicity.

Pharmacodynamics 1: effect on SERCA2a and Na^+/K^+ ATPase activities in cell-free preparations (enzymatic assays)

Renal Na^+/K^+ ATPase purification and activity. Purification of renal Na^+/K^+ ATPase was performed according to the method of Jørgensen (Jørgensen, 1988). Frozen kidneys from 1-3 years-old male beagle dogs were obtained from the General Pharmacology Department of Sigma-tau, Pomezia, Italy. Kidneys were sliced and the outer medulla was dissected, pooled and suspended in a sucrose-histidine solution, containing 250 mM sucrose, 30 mM histidine and 5 mM EDTA, pH 7.2 and homogenized. The homogenate was centrifuged at 6,000 g for 15 min, the supernatant was decanted and centrifuged at 48,000 g for 30 min. The pellet was suspended in the sucrose-histidine buffer and incubated for 20 min with a sodium-dodecyl-sulphate (SDS) solution, dissolved in a gradient buffer, containing 25 mM imidazole and 1 mM EDTA, pH 7.5. The sample was layered on the top of a sucrose discontinuous gradient (10, 15 and 29.4%) and centrifuged at 60,000 g for 115 min. The final pellet was suspended in the gradient buffer. Na^+/K^+ ATPase activity was assayed by measuring ^{32}P-ATP hydrolysis, as previously described (Ferrandi *et al.*, 1996). Increasing concentrations of the standard ouabain, or tested compound, were incubated with 0.3 μg of purified dog kidney enzyme for 10 min at 37°C in 120 μl final volume of a medium, containing 140 mM NaCl, 3 mM $MgCl_2$, 50 mM Hepes-Tris, 3 mM ATP, pH 7.5. Then, 10 μl of incubation solution containing 10 mM KCl and 20 nCi of ^{32}P-ATP (3-10 Ci/mmol, Perkin Elmer) were added, the reaction continued for 15 min at 37°C and was stopped by acidification with 20% ice-cold perchloric acid. ^{32}P was separated by centrifugation with activated Charcoal (Norit A, Serva) and the radioactivity was measured. Effects of increasing concentrations of the test compound were compared to ouabain, as positive standard, and to vehicle (control) at 37°C. The inhibitory activity was expressed as percent of activity in control.

SERCA ATPase activity assay. LV were dissected from rat and guinea pig of healthy and failing preparations and frozen until use. Tissues were homogenized, subjected to centrifugation to obtain SR-enriched microsomes and sarcomeric proteins were extracted, as previously described (Micheletti *et al.*, 2007). In the case of rat hearts, cardiac homogenates were used in order to have sufficient material to replicate the experiments within a single animal. LV tissues from healthy and STZ rats were homogenized in a medium containing 300 mM sucrose, 50 mM K-phosphate, 10 mM NaF, 0.3 mM PMSF, 0.5 mM DTT (pH 7) and centrifuged at 35,000 g for 30 min. The final pellet was resuspended in the same buffer. In the case of guinea-pig hearts, LV tissues were homogenized in 4 volumes of 10 mM $NaHCO_3$, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin (pH 7) and centrifuged at 12,000g for 15 minutes. Supernatants were filtered and centrifuged at 100,000 g for 30 min. Contractile proteins were extracted by suspending the pellets with 0.6 M KCl, 30 mM histidine, pH 7 and further centrifugation at 100,000 g for 30 min. Final pellets were reconstituted with 0.3 M sucrose, 30 mM histidine, pH 7, to obtain SR-enriched microsomes. SERCA2a ATPase activity was measured as the rate of ^{32}P-ATP release at multiple Ca^{2+} concentrations in the absence and presence of test compounds, as previously described (Micheletti *et al.*, 2007). Increasing concentrations of each compound were pre-incubated with 2 μg of cardiac preparations for 5 min at 4° C in 80 μl of a solution containing 100

mM KCl, 5 mM $MgCl_2$, 1 µM A23187, 20 mM Tris, pH 7.5. Then, 20 µl of 5 mM Tris-ATP containing 50 nCi of ^{32}P-ATP (3-10 Ci/mmol, Perkin Elmer) were added. The ATP hydrolysis was continued for 15 min at 37°C and the reaction was stopped by acidification with 100 µl of 20% ice-cold perchloric acid. ^{32}P was separated by centrifugation with activated charcoal (Norit A, Serva) and the radioactivity was measured.

Reconstitution of SERCA1 with PLN_{1-32} synthetic fragment. Adult healthy guinea-pigs were used to prepare SERCA1-enriched SR microsomes from fast-twitch hind leg muscles. Microsomes were prepared as described for SERCA2a preparations. For reconstitution experiments, SERCA1 (PLN-free) was pre-incubated with synthetic PLN_{1-32} fragment (canine sequence, Biomatik Corporation, Canada) in 20 mM imidazole, pH 7, at 1:300 SERCA1:PLN ratio for 30 min at room temperature. After pre-incubation, SERCA1 (PLN-free) alone, or reconstituted with PLN_{1-32} fragment, was utilized for SERCA activity measurement by using ^{32}P-ATP hydrolysis method at different Ca^{2+} concentrations in the absence and presence of increasing concentrations of tested compounds, as described for SERCA2a ATPase activity.

Pharmacodynamics 2: in-vitro effects for ligands potentially accounting for off-target actions

Analysis of PST3093 interaction with a panel of 50 ligands was carried out by Eurofins (Taiwan) on crude membrane preparations according to Eurofins described procedures. The assay is partly based on radioligand displacement (e.g. for receptors) and partly on spectrophotometric detection of change in function (e.g. for enzymes). Results were compared to appropriate reference standards; a >50% change in affinity or activity was considered as a positive hit (interaction present).

Pharmacodynamics 3: effect on Na^+/K^+ ATPase current and intracellular Ca^{2+} dynamics

Rat and guinea-pig LV ventricular myocytes were isolated by using a retrograde coronary perfusion method previously published (Rocchetti *et al.*, 2003) with minor modifications. Rod-shaped, Ca^{2+}-tolerant myocytes were used within 12 h from dissociation. LV myocytes were clamped in the whole-cell configuration (Axopatch 200A, Axon Instruments Inc., Union City, CA). During measurements, myocytes were superfused at 2 ml/min with Tyrode's solution containing 154 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES/NaOH, and 5.5 mM D-glucose, adjusted to pH 7.35. A thermostated manifold, allowing for fast (electronically timed) solution switch, was used for cell superfusion. All measurements were performed at 35 °C. The pipette solution contained 110 mM K^+ -aspartate, 23 mM KCl, 0.2 mM $CaCl_2$ (10^{-7} M calculated free-Ca^{2+} concentration), 3 mM $MgCl_2$, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 0.4 mM GTP-Na^+ salt, 5 mM ATP-Na^+ salt, and 5 mM creatine phosphate Na^+ salt, pH 7.3. Membrane capacitance and series resistance were measured in every cell but left uncompensated. Current signals were filtered at 2 KHz and digitized at 5 KHz (Axon Digidata 1200). Trace acquisition and analysis was controlled by dedicated software (Axon pClamp 8.0).

Na^+/K^+ ATPase current (I_{NaK}) measurements. I_{NaK} was recorded in isolated rat LV myocytes (Rocchetti *et al.*, 2003; Alemanni *et al.*, 2011) as the holding current recorded at -40 mV in the presence of Ni^{2+} (5 mM), nifedipine (5 µM), Ba^{2+} (1 mM) and 4-aminopyridine (2 mM) to minimize contamination by changes in Na^+/Ca^{2+} exchanger (NCX), Ca^{2+} and K^+ currents, respectively. Tetraethylammonium-Cl (20 mM) and EGTA (5 mM) were added to the pipette solution and intracellular K^+ was replaced by Cs^+. To optimize the recording conditions, I_{NaK} was enhanced by increasing intracellular Na^+ (10 mM) and extracellular K^+ (5.4 mM). All drugs were dissolved in dimethyl sulfoxide (DMSO). Control and test solutions contained maximum 1:100 DMSO.

Intracellular Ca^{2+} dynamics. LV myocytes were incubated in Tyrode's solution for 30 min with the membrane-permeant form of the dye, Fluo4-AM (10 µM), and then washed for 15 min to allow dye de-esterification. Fluo4 emission was collected through a 535 nm band pass filter, converted to voltage, low-pass filtered (100 Hz) and digitized at 2 kHz after further low-pass digital filtering (FFT, 50 Hz). After subtraction of background luminescence, a reference fluorescence (F_0) value was used for signal normalization (F/F_0). Cytosolic Ca^{2+} activity was dynamically measured in field stimulated (2 Hz) and patch-clamped rat LV myocytes. In the first case, fluorescence in diastole was used as F_0 for signal normalization (F/F_0).

In patch-clamped myocytes membrane current, whose time-dependent component mainly reflected the sarcolemmal Ca^{2+} current (I_{CaL}), was simultaneously recorded. Drug effects on SR Ca^{2+} uptake rate were evaluated with a "SR loading protocol" specifically devised to rule out the contribution of NCX and to assess the SR Ca^{2+} uptake rate at multiple levels of SR Ca^{2+} loading (Rocchetti *et al.*, 2005) (protocol in Figure S1). The protocol consisted in emptying the SR by a brief caffeine (10 mM) pulse and then progressively refilling it by 7-10 voltage steps (-35 to 0 mV) activating Ca^{2+} influx through I_{CaL}. NCX was blocked by omission of Na^+ from intracellular and extracellular (replaced by equimolar Li^+ and 1 mM EGTA) solutions. The procedure is in agreement with published methods, with minor modifications (Rocchetti *et al.*, 2005; Alemanni *et al.*, 2011; Torre *et al.*, 2021). Multiple parameters, suitable to quantify SR Ca^{2+} uptake, can be extracted from Ca^{2+} and I_{CaL} response to the protocol: the time constant (τ) of cytosolic Ca^{2+} decay within each V-step largely reflects net Ca^{2+} flux across the SR membrane (the faster SR Ca^{2+} uptake, the smaller τ decay). Because of the steep dependency of CaT amplitude on SR Ca^{2+} content, the rate at which CaT amplitude increases across the subsequent pulses of the protocol reflects the rate at which the SR refills. To rule out the potential contribution of changes in I_{CaL}, in each loading step, CaT amplitude was normalized to Ca^{2+} influx (estimated from I_{CaL} integral up to CaT peak) to obtain excitation-release (ER) gain. As expected from its strong dependency on SR Ca^{2+} content, this parameter progressively increases during the loading protocol. Diastolic Ca^{2+} of the first step was used as F_0 for signal normalization (F/F_0). Specificity of the "loading protocol" parameters in detecting SERCA2a activation is supported by the observation that they did not detect any effect of digoxin, an inotropic agent blocking the Na^+/K^+ pump and devoid of SERCA2a stimulating effect (Rocchetti *et al.*, 2005; Alemanni *et al.*, 2011).

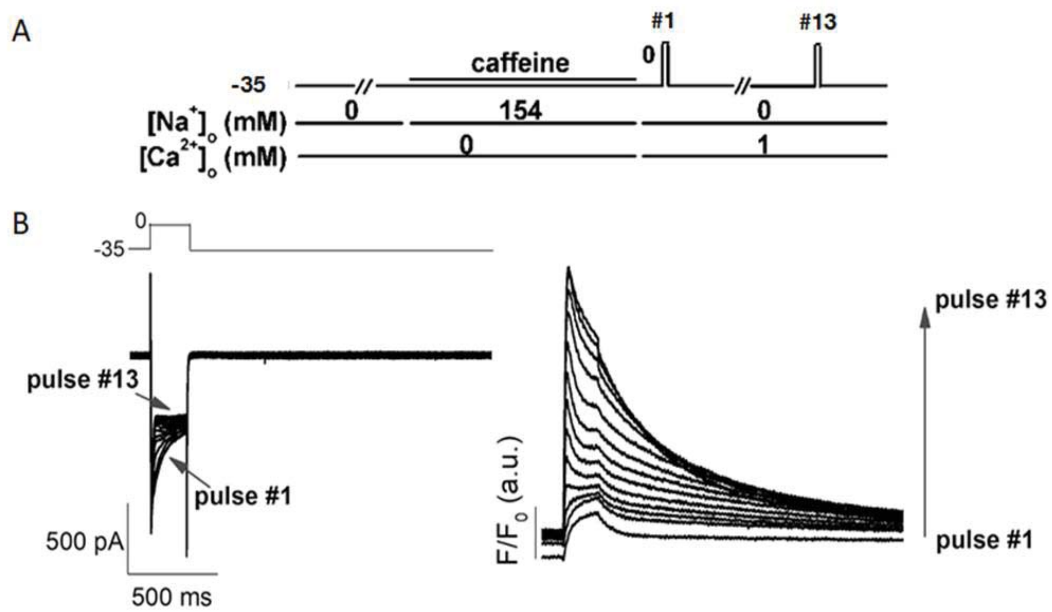
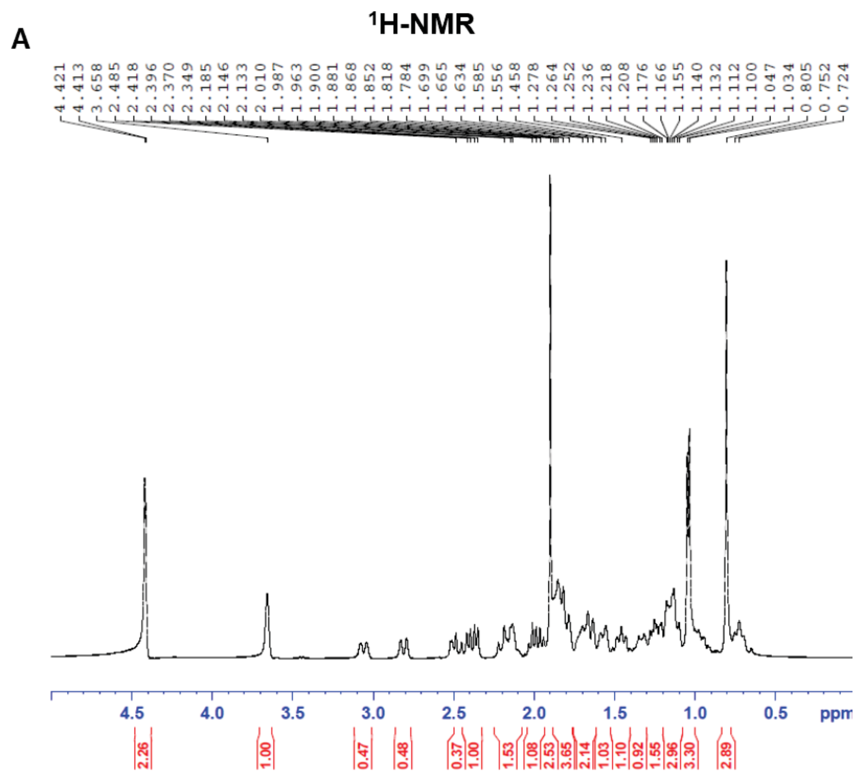
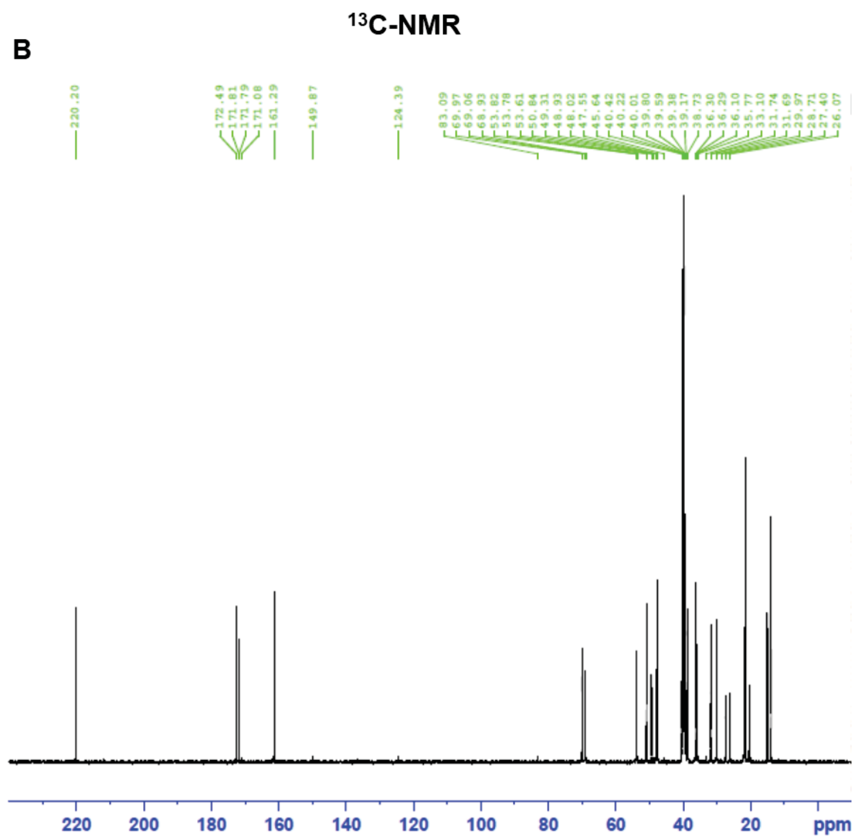
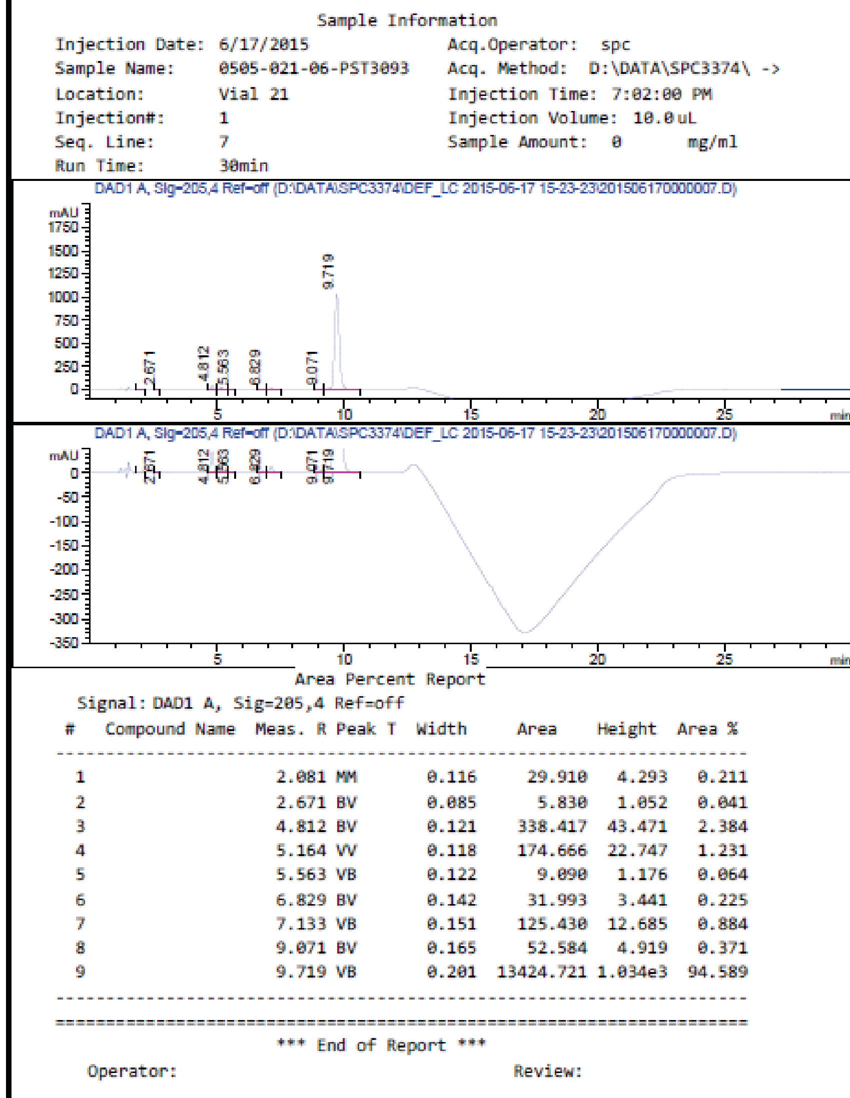


Figure S1. Protocol to evaluate intracellular Ca^{2+} dynamics in patch-clamped cells under Na^{+} free condition. **A)** Protocol outline. **B)** Transmembrane current (left) and Ca^{2+} transients (right) recordings during SR reloading after caffeine-induced SR depletion in patch-clamped cells. See Methods for details.


A
1H-NMR
4.421 4.413 3.658 2.485 2.418 2.396 2.370 2.349 2.185 2.146 2.133 2.010 1.987 1.963 1.900 1.881 1.868 1.852 1.818 1.784 1.699 1.665 1.634 1.585 1.556 1.458 1.278 1.264 1.252 1.236 1.218 1.208 1.176 1.166 1.155 1.140 1.132 1.112 1.100 1.047 1.034 0.805 0.752 0.724
4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
2.26 1.00 0.47 0.48 0.37 1.00 1.53 1.08 2.53 3.65 2.14 1.03 1.10 0.92 1.55 2.96 3.30 2.89


B
13C-NMR
220 200 180 160 140 120 100 80 60 40 20 ppm

C



#	Compound Name	Meas. R	Peak T	Width	Area	Height	Area %
1		2.081	MM	0.116	29.910	4.293	0.211
2		2.671	BV	0.085	5.830	1.052	0.041
3		4.812	BV	0.121	338.417	43.471	2.384
4		5.164	VV	0.118	174.666	22.747	1.231
5		5.563	VB	0.122	9.090	1.176	0.064
6		6.829	BV	0.142	31.993	3.441	0.225
7		7.133	VB	0.151	125.430	12.685	0.884
8		9.071	BV	0.165	52.584	4.919	0.371
9		9.719	VB	0.201	13424.721	1.034e3	94.589

Figure S2. Analytical characterization of PST3093. A) ^{1}H-NMR in DMSO (400 MHz, Bruker), **B)** ^{13}C-NMR in DMSO (100 MHz, Bruker), **C)** HPLC profile (about 95% purity).

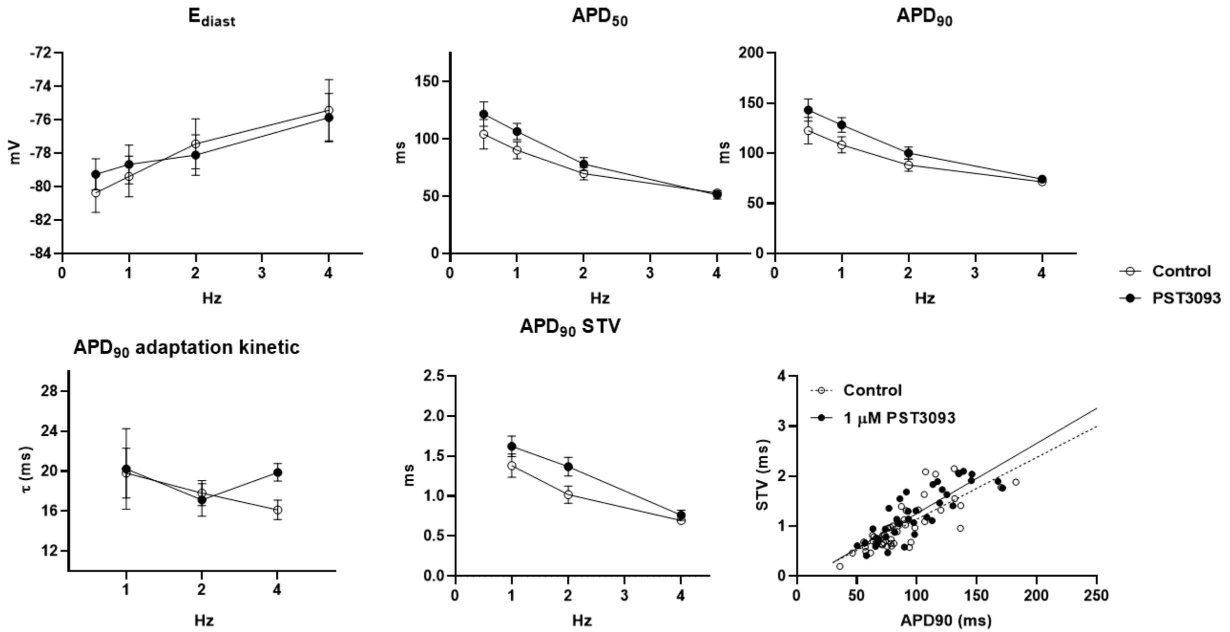


Figure S3. Effects of 1 µM PST3093 on electrical activity in guinea-pig myocytes. Rate dependency of AP parameters (E_{diast}, APD_{50}, APD_{90}), APD_{90} adaptation kinetics and APD_{90} short term variability (STV) with or w/o 1 µM PST3093; N=3 (n=16 w/o PST3093, n=18 with PST3093). Bottom right: linear correlation between STV of APD_{90} and APD_{90} values with or w/o PST3093; data from 1, 2, 4 Hz were pooled (w/o PST3093 slope = 0.012, with PST3093 slope = 0.014, NS).

Table S1. Effect of PST3093 and istaroxime on SERCA2a kinetic parameters in cardiac preparations from healthy guinea-pigs. Data are mean ± SEM; N indicates the number of experiments. *$p<0.05$ vs control (RM one-way ANOVA with post-hoc Tukey's multiple comparisons test or paired *t*-test).

<table>
<tr><th colspan="7">Guinea-pigs</th></tr>
<tr><th>[nM]</th><th>N</th><th>control</th><th>PST3093</th><th>N</th><th>control</th><th>istaroxime</th></tr>
<tr><td colspan="7">V_{max} (µmol/min/mg)</td></tr>
<tr><td>1</td><td rowspan="2">9</td><td rowspan="2">2,28±0,17</td><td>2,16±0,18</td><td rowspan="3">9</td><td rowspan="3">2,51±0,08</td><td>2,50±0,04</td></tr>
<tr><td>10</td><td>2,15±0,15</td><td>2,47±0,06</td></tr>
<tr><td>100</td><td>11</td><td>2,38±0,16</td><td>2,34±0,16</td><td>2,49±0,07</td></tr>
<tr><td colspan="7">K_dCa (nM)</td></tr>
<tr><td>1</td><td rowspan="2">9</td><td rowspan="2">567±12</td><td>504±23*</td><td rowspan="3">9</td><td rowspan="3">584±22</td><td>524±19</td></tr>
<tr><td>10</td><td>501±17*</td><td>485±16*</td></tr>
<tr><td>100</td><td>11</td><td>557±12</td><td>445±30*</td><td>478±19*</td></tr>
</table>

Table S2. Effect of PST3093 (10 µM) on the panel of molecular targets (Eurofins, Taiwan).

	Cat #	Assay name	Batch	Species	PST3093 effect (%)
1	107480	ATPase, Ca^{2+}, skeletal muscle	438642	pig	-18
2	118040	CYP450, 19	438644	human	12
3	124010	HMG-CoA Reductase	438610	human	-2
4	140010	Monoamine Oxidase MAO-A	438645	human	2
5	140120	Monoamine Oxidase MAO-B	438647	human	-3
6	143000	Nitric Oxide Synthase, Endothelial (eNOS)	438568	bovine	2
7	107300	Peptidase, Angiotensin Converting Enzyme	438641	rabbit	3
8	164610	Peptidase, Renin	438648	human	6
9	152000	Phosphodiesterase PDE3	438611	human	-3
10	171601	Protein Tyrosine Kinase, ABL1	438612	human	3
11	176810	Protein Tyrosine Kinase, Src	438613	human	-1
12	200510	Adenosine A1	438614	human	-2
13	200610	Adenosine A2A	438614	human	-6
14	203100	Adrenergic α1A	438615	rat	2
15	203200	Adrenergic α1B	438615	rat	0
16	203630	Adrenergic α2A	438616	human	-5
17	204010	Adrenergic β1	438652	human	-4
18	204110	Adrenergic β2	438571	human	7
19	204600	Aldosterone	438617	rat	5
20	206000	Androgen (Testosterone)	438618	human	3
21	210030	Angiotensin AT1	438653	human	-1
22	210120	Angiotensin AT2	438653	human	7
23	214600	Calcium Channel L-type, Dihydropyridine	438620	rat	-8
24	219500	Dopamine D1	438660	human	3

25	219700	Dopamine D2s	439024	human	26
26	219800	Dopamine D3	438660	human	1
27	226010	Estrogen ERα	438622	human	-6
28	226050	Estrogen ERβ	438622	human	7
29	226600	$GABA_A$, Flunitrazepam, Central	438624	rat	4
30	226500	$GABA_A$, Muscimol, Central	438623	rat	9
31	232030	Glucocorticoid	438626	human	8
32	233000	Glutamate, NMDA, Phencyclidine	438627	rat	-8
33	239610	Histamine H1	438628	human	-3
34	241000	Imidazoline I2, Central	438629	rat	5
35	243000	Insulin	438654	rat	6
36	252710	Muscarinic M2	438621	human	-7
37	252810	Muscarinic M3	438661	human	4
38	253010	Muscarinic M5	438661	human	5
39	258730	Nicotinic Acetylcholine α3β4	438656	human	1
40	260410	Opiate μ (OP3, MOP)	438616	human	-5
41	264500	Phorbol Ester	438624	mouse	-7
42	265600	Potassium Channel (K_{ATP})	438632	hamster	-8
43	265900	Potassium Channel hERG	438633	human	10
44	299005	Progesterone PR-B	438638	human	-15
45	270300	Ryanodine RyR3	438634	rat	2
46	271010	Serotonin (5-Hydroxytryptamine) 5-HT1, non-selective	438668	rat	0
47	299007	Sigma σ2	438662	human	12
48	278110	Sigma σ1	438636	human	7
49	279510	Sodium Channel, Site 2	438637	rat	2
50	204410	Transporter, Norepinephrine (NET)	438597	human	4

Table S3. Characterization of the STZ diabetic rat model. Parameters were measured after 1 or 8 weeks (wk) from STZ injection. Data are mean ± SEM. N represents the number of rats for each group. *p<0.05 vs healthy rats (unpaired *t*-test).

	Healthy		**STZ**	
Parameters	**1 wk after vehicle**	**8 wk after vehicle**	**1 wk after STZ**	**8 wk after STZ**
BW (g)	201±4.7	449±11.8	193±4.2	305±12.9*
Glycaemia (mg/dL)	130±6.6	nd	398±16*	nd
LV mass (mm^3)	nd	985±34	nd	781±40*
LV mass index (mm^3/g)	nd	2,23±0,1	nd	2,66±0,2
N	18	18	20	20

Table S4. Echocardiographic parameters in healthy and STZ diabetic rats. Data are mean ± SEM. N represents the number of rats for each group. *p<0.05 vs healthy rats (unpaired *t*-test).

Echo parameters		Healthy	STZ
Morphometric parameters	IVSTd (mm)	2,22±0,07	1,73±0,1 *
	PWTd (mm)	1,87±0,09	1,54±0,05 *
	LVEDD (mm)	6,6±0,14	6,95±0,13
	IVSTs (mm)	2,46±0,07	2,26±0,1
	PWTs (mm)	2,96±0,09	2,47±0,11 *
	LVESD (mm)	3,09±0,09	3,54±0,1 *
Systolic parameters	FS (%)	53,08±1,17	48,73±1,37 *
	s' (mm/s)	29,59±1,15	24,8±0,75 *
	EF (%)	88 ± 0,8	85 ±1,08 *
Diastolic parameters	E (mm/s)	0,93±0,01	0,84±0,02 *
	A (mm/s)	0,65±0,04	0,63±0,03
	E/A	1,50±0,08	1,38±0,07
	DT (ms)	54,61±2,1	56,8±2,16
	DT/E	59,23±2,4	68,37±3,0 *
	E/DT	17,41±0,77	15,18±0,67 *
	e' (mm/s)	23,46±0,77	21,34±0,58 *
	a' (mm/s)	24,54±1,38	24,3±1,13
	e'/a'	1,01±0,06	0,91±0,04
	E/e'	40,1±1,33	39,64±1,0
Overall cardiac function	HR (bpm)	306±10	248±7,4 *
	LV EDV (mL)	0.67±0.04	0.77±0.04
	LV ESV (mL)	0.08±0.01	0.12±0.01 *
	SV (ml)	0,59±0,03	0,65±0,04
	CO (ml/min)	178,9±10,2	161,1±10,9
	N	18	20

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