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ORIGINAL ARTICLE

SEA0400 fails to alter the magnitude of intracellular Ca^{2+} transients and contractions in Langendorff-perfused guinea pig heart

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Abstract SEA0400 is a recently developed inhibitor of the Na^+/Ca^{2+} exchanger (NCX) shown to suppress both forward and reverse mode operation of NCX. Present experiments were designed to study the effect of partial blockade of NCX on Ca handling and contractility in Langendorff-perfused guinea pig hearts loaded with the fluorescent Ca-sensitive dye fura-2. Left ventricular pressure and intracellular calcium concentration ($[Ca^{2+}]_i$) were synchronously recorded before and after cumulative superfusion with 0.3 and 1 μM SEA0400. SEA0400 caused no significant change in the systolic and diastolic values of left ventricular pressure and $[Ca^{2+}]_i$. Accordingly, pulse pressure and amplitude of the $[Ca^{2+}]_i$ transient also remained unchanged in the presence of SEA0400. SEA0400 had no influence either on the time required to reach peak values of pressure and $[Ca^{2+}]_i$ or on half relaxation time. On the other hand, both 0.3 and 1 μM SEA0400 significantly increased the decay time constant of $[Ca^{2+}]_i$ transients, obtained by fitting its descending limb between 30% and 90% of relaxation, from 127±7 to 165±7 and 177±14 ms, respectively ($P<0.05$, $n=6$). In contrast to the guinea pig hearts, rat hearts responded to SEA0400 treatment with increased $[Ca^{2+}]_i$ transients and contractility. These interspecies differences observed in the effect of SEA0400 can be explained by the known differences in calcium handling between the two species.

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Introduction

Na^+/Ca^{2+} exchange (NCX) is a crucial mechanism of Ca^{2+} extrusion from the myocardium in various mammalian species and as such is involved in the control of cardiac contraction (Bers 2000; Reuter et al. 2005). In the forward mode of action, it is responsible for the extrusion of Ca^{2+} that enters the cell during each cardiac cycle but also contributes to Ca^{2+} influx in the early phase of systole when working in a reverse mode (Bers 2000). Selective inhibition of NCX has important therapeutic implications since it was reported to prevent early and delayed after-depolarizations (Watano et al. 1999; Nagy et al. 2004), to restore the impaired Ca^{2+} handling in heart failure (Hobai et al. 2004), and to reduce the ischemia/reperfusion-induced cellular injuries and arrhythmias (Yamamura et al. 2001; Takahashi et al. 2003). Major objection against the therapeutic application of NCX blockers is based on the assumption that they might elevate cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)—as can be logically expected if NCX is the dominant mechanism of Ca^{2+} extrusion (Sipido et al. 2006). Indeed, the NCX blocker SEA0400 was shown to increase $[Ca^{2+}]_i$ and contractility significantly in murine and rat myocardium (Tanaka et al. 2005; Acsai et al. 2007). In contrast, SEA0400 increased the force of contraction by only 5% or less in guinea pig ventricular myocardium (Namekata et al. 2005; Tanaka et al. 2007), suggesting that the inhibition of NCX may have less dramatic consequences in this species. In the present work, therefore, the effect of SEA0400 on intracellular Ca^{2+} handling and contractility

was studied in Langendorff-perfused guinea pig hearts, which allows the investigation of NCX inhibition under conditions close to physiological (i.e., when the tissue structure and transmembrane ion channels and transporters are intact and $[Ca^{2+}]_i$ is not buffered by ethylene glycol tetraacetic acid). We found that SEA0400 failed to affect the magnitude of $[Ca^{2+}]_i$ transients and contractility in this preparation. These results are consistent with a limited inhibitory effect of SEA0400 on the forward mode activity of NCX under physiological conditions. This information is crucial regarding the potential therapeutic application of the compound.

Materials and methods

Effects of SEA0400 on $[Ca^{2+}]_i$ transients and contractilty were studied in six Langendorff-perfused guinea pig hearts. The animals (weighing 300–500 g) were intraperitoneally heparinized and anesthetized with Na-pentobarbital (150 mg/kg). After the opening of the animal's chest, the heart was rapidly removed and fixed to the cannula of a Langendorff-perfusion device. The heart was perfused with a modified Kreb's solution containing NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, Na_2EDTA 0.5, KH_2PO_4 0.23, and glucose 5.5 mM. The pH of this perfusate was set to 7.4 when gassed with a mixture of 5% CO_2 and 95% O_2 at 37°C. The coronary flow rate, controlled by a peristaltic pump, was adjusted to 10 ml min^{-1} g^{-1}. Left ventricular pressure (LVP) was continuously monitored using a Braun 2021-02 arterial pressure transducer that was connected to the left ventricular cavity (Edes and Kranias 1990). The heart rate was maintained at 200 beats per minute by left atrial pacing.

To record $[Ca^{2+}]_i$ transients, the heart was loaded with the acetoxymethylester form of the fluorescent dye, fura-2 (5 μM), added to the perfusate together with probenecid (0.6 mM), synperonic (1.25 g/l), and albumin (50 g/l). Probenecid was used to inhibit the unspecific anion exchanger of the cell membrane to avoid the extrusion of fura-2 from the cells. Synperonic and albumin enhanced loading with fura-2-AM. Due to the presence of probenecid, peak LVP was lower than normal, but under these conditions, stable calcium signals were obtained for periods as long as 120 min (Kristof et al. 1998). Fura-2 was excited at both 340- and 380-nm wavelengths. The emitted light was collected at 510 nm using a trifurcated quartz fiber optic bundle connected to a Deltascan device (Photon Technology International, New Brunswick, NJ, USA). $[Ca^{2+}]_i$ was measured as a fluorescent ratio (F_{340}/F_{380}). The analogue signals of fluorescence and LVP were sampled at 1 kHz. In each case, ten subsequent beats were averaged and stored for analysis.

After stabilization of the contractile parameters and $[Ca^{2+}]_i$ transients, 25 min of control recording was performed in Kreb's solution. Then, SEA0400 was applied in a cumulative manner: 0.3- and 1-μM concentrations were consecutively perfused, each for 25 min. SEA0400 was dissolved in dimethyl sulfoxide (DMSO) so as having a stock solution of 3 mM. Appropriate quantities of this stock solution were added to the bath yielding 0.3- or 1-μM final concentrations of SEA0400. All solutions contained 0.033% DMSO. This low concentration of DMSO had no effect on the parameters studied.

The effects of 0.3 and 1 μM SEA0400 were assessed also in Langendorff-perfused rat hearts. In these experiments, the ratiometric fluorescent dye, indo-1, was applied to record intracellular Ca^{2+} transients. The hearts were loaded with 1 μM indo-1-AM for 30 min. The dye accumulated in the left ventricular wall was excited at 355 nm, and the fluorescence emission of the saturated and free Ca^{2+} levels were detected at 400 and 500 nm after correction for nonspecific background fluorescence. $[Ca^{2+}]_i$ was expressed as a fluorescent ratio (F/F_0). The effects of SEA0400 in these rat hearts were studied in a noncumulative manner following a 20-min period of incubation with concentrations of 0.3 (n=6) and 1 μM (n=8).

Results are expressed as mean±SEM values. Statistical significance of differences was evaluated with using one-way analysis of variance followed by Dunnett's test. Differences were considered significant when P was less than 0.05.

Results

The effect of 25-min consecutive perfusion with 0.3 and 1 μM of SEA0400 was studied in six Langendorff-perfused guinea pig hearts. As shown by the analogue records presented in Fig. 1a, SEA0400 had no obvious effect on the LVP in these preparations. No significant change was observed in the systolic pressure (55.5±2.7 mmHg in control versus the 58.6±3.5 and 53.0±3.9 mmHg measured at the end of 0.3 and 1 μM SEA0400 perfusion, respectively), end-diastolic pressure (6.0±0.2 vs. 6.8±0.4 and 6.4±0.4 mmHg), and pulse pressure (49.5±2.8 vs. 51.8±3.5 and 46.6±3.8 mmHg). The fluorescent ratio, used as indicator of $[Ca^{2+}]_i$, was also unchanged by the SEA0400 perfusion, as shown in Fig. 2b. The respective values for the peak fluorescent ratio were 1.04±0.01 vs. 1.04±0.02 and 1.05±0.03, for the baseline fluorescent ratio: 0.81±0.04 vs. 0.81±0.04 and 0.83±0.04, and for fluorescent ratio, amplitude values of 0.23±0.03 vs. 0.22±0.03 and 0.22±0.03 were obtained. The changes of these parameters as a function of time are presented in Fig. 2.

Kinetic properties of pressure changes were characterized by time required to develop peak pressure (TtP), time

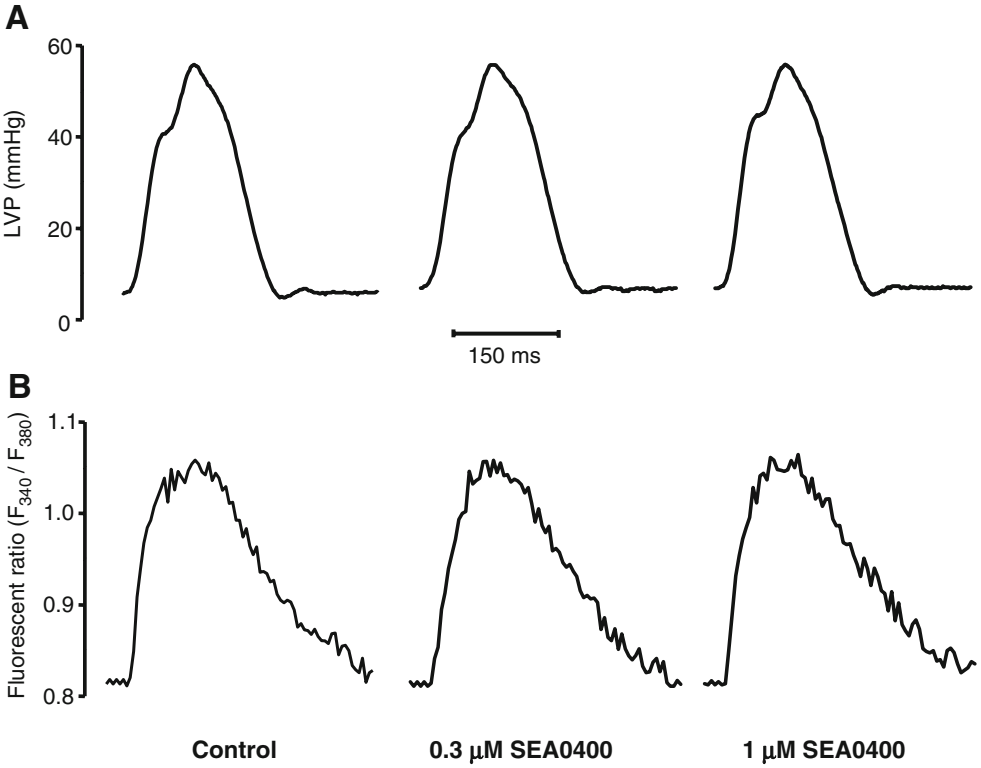

Fig. 1 Representative set of analogue records showing the changes in left ventricular pressure (*LVP*, **a**) and cytosolic free calcium concentration (expressed as a fluorescent ratio of F_{340}/F_{380}, **b**) in a Langendorff-perfused guinea pig heart before and after 25 min cumulative perfusion with 0.3 and 1 μM SEA0400

required to achieve half relaxation (HRT), and by the maximum and minimum values of first time derivatives of the pressure (+d*P*/d*t*, −d*P*/d*t*) obtained during systole and diastole, respectively. No significant changes were observed in any of these parameters (Fig. 3a,b). The $[Ca^{2+}]_i$ transients obtained before and after SEA0400 treatment were analyzed in a similar manner, except for a segment of the decaying phase of the curve (from 30% to 90% of relaxation), which was fitted to a monoexponential function. These results are presented in Fig. 3c,d. SEA0400

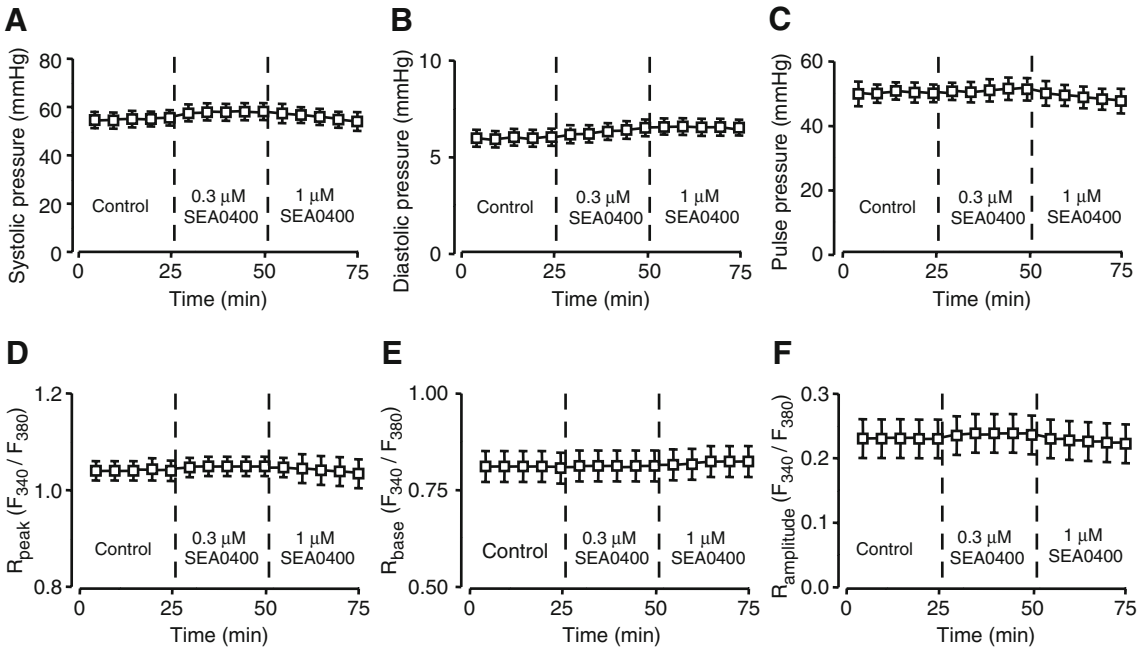

Fig. 2 Effect of 0.3 and 1 μM SEA0400 on systolic (**a**), end-diastolic (**b**), and pulse pressure (**c**) as a function of time. *Lower panels* (**d**, **e**, **f**) show parameters of the concomitantly recorded $[Ca^{2+}]_i$ transients: fluorescent ratios measured during systole, diastole, and their difference (R_{peak} **a**, R_{base} **b**, and $R_{amplitude}$ **c**, respectively). *Symbols* and *bars* represent mean values±SEM obtained in six hearts

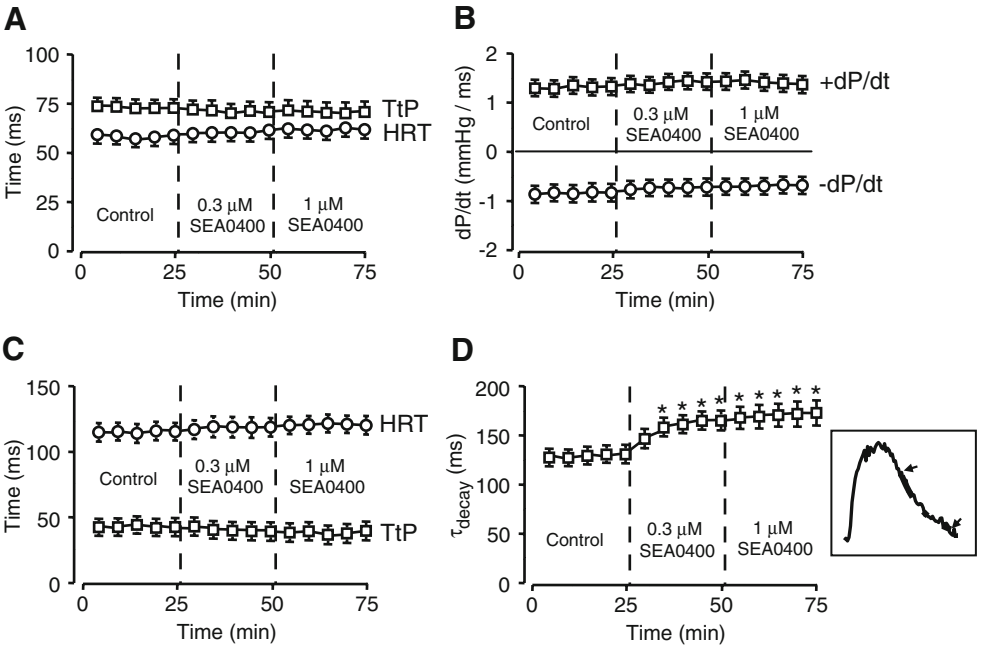

Fig. 3 Kinetic properties of changes in left ventricular pressure (**a**, **b**) and $[Ca^{2+}]_i$ transients (**c**, **d**) in the absence and presence of SEA0400. *TtP* Time required to develop peak pressure (**a**) or peak $[Ca^{2+}]_i$ (**c**), *HRT* half relaxation time of pressure (**a**) or $[Ca^{2+}]_i$ (**c**). Maximum and minimum values of first time derivatives of the pressure (+d*P*/d*t* and −d*P*/d*t*, obtained during systole and diastole, respectively) are depicted in **b**. **d** Decay time constant of $[Ca^{2+}]_i$ transients obtained by monoexponential fit (from 30% to 90% of relaxation, as shown in the *inset*). *Symbols* and *bars* represent means±SEM obtained in six hearts; *asterisks* indicate significant changes from control values, measured just prior to SEA0400 treatment

caused no significant change in the TtP and HRT values obtained for the fluorescent ratio; however, the decay time constant was significantly increased by 0.3 and 1 µM SEA0400 (from 127±7 to 165±7 and 177±14 ms, respectively, $P<0.05$).

The effect of 0.3 and 1 µM of SEA0400 was studied in Langendorff-perfused rat hearts as well. As shown in Fig. 4a–f, the developed pressure and the amplitude of the $[Ca^{2+}]_i$ transient was significantly increased following 20-min perfusion with 0.3 and 1 µM SEA0400 studied in six and eight rat hearts, respectively. These results indicate that SEA0400 was able to increase the amplitude of $[Ca^{2+}]_i$ transient and contractility in rat but not in guinea pig ventricular myocardium. Another set of positive control experiments is presented in Fig. 5a–f, where a guinea pig heart was exposed to 100 nM isoproterenol for 1 min. Isoproterenol strongly increased the amplitude of both $[Ca^{2+}]_i$ transient and the developed pressure; in addition, it enhanced the rate of contraction and relaxation—as it could be expected in case of a preparation working under reasonably good metabolic conditions. Similar responses were observed whenever guinea pig hearts were challenged by 100 nM isoproterenol.

Discussion

The major finding of the present study was that SEA0400-induced partial NCX blockade failed to alter the developed pressure and $[Ca^{2+}]_i$ transients in Langendorff-perfused guinea pig hearts. This is apparently in sharp contrast with the present results obtained with Langendorff-perfused rat hearts and previous observations in rat and murine ventricular cardiomyocytes, where SEA0400 increased both contractility and $[Ca^{2+}]_i$ transients (Acsai et al. 2007; Tanaka et al. 2005). This paradoxical situation can be resolved by considering the differences in the conditions that determine the operation of NCX, i.e., the actual values of transmembrane potential and cytosolic free sodium and calcium ion concentrations (Blaustein and Lederer 1999). Action potential duration is much shorter (50 versus 250 ms), and intracellular sodium concentration is much higher (15 versus 8 mM) in rats and mice than in other mammalian species including guinea pig (Bers 2001). Therefore, NCX operates dominantly in the forward mode during systole in rats and mice, while the longer action potential duration and lower intracellular sodium concentration allow NCX to operate in the reverse mode for a longer period of time during early systole in guinea pigs (Bers 2001). As a consequence, symmetrical NCX inhibition (i.e., equal fractional blockade of the forward and reverse mode operation) is expected to cause a greater increase in $[Ca^{2+}]_i$ in rats or mice than in guinea pigs. On the other hand, since SEA0400 was shown to inhibit the reverse mode operation of NCX more effectively than its forward mode activity (Lee et al. 2004), SEA0400 is likely to suppress calcium influx via NCX more effectively in guinea pigs than in rats and mice. This favors calcium accumulation in SEA0400-treated rat and murine hearts in contrast to guinea pig myocardium. Similarly to the present observations, SEA0400 had no effect on contractility in anesthetized dogs (Takahashi et al. 2004). These results together suggest that NCX inhibition may increase $[Ca^{2+}]_i$ and contractility under physiological conditions only in

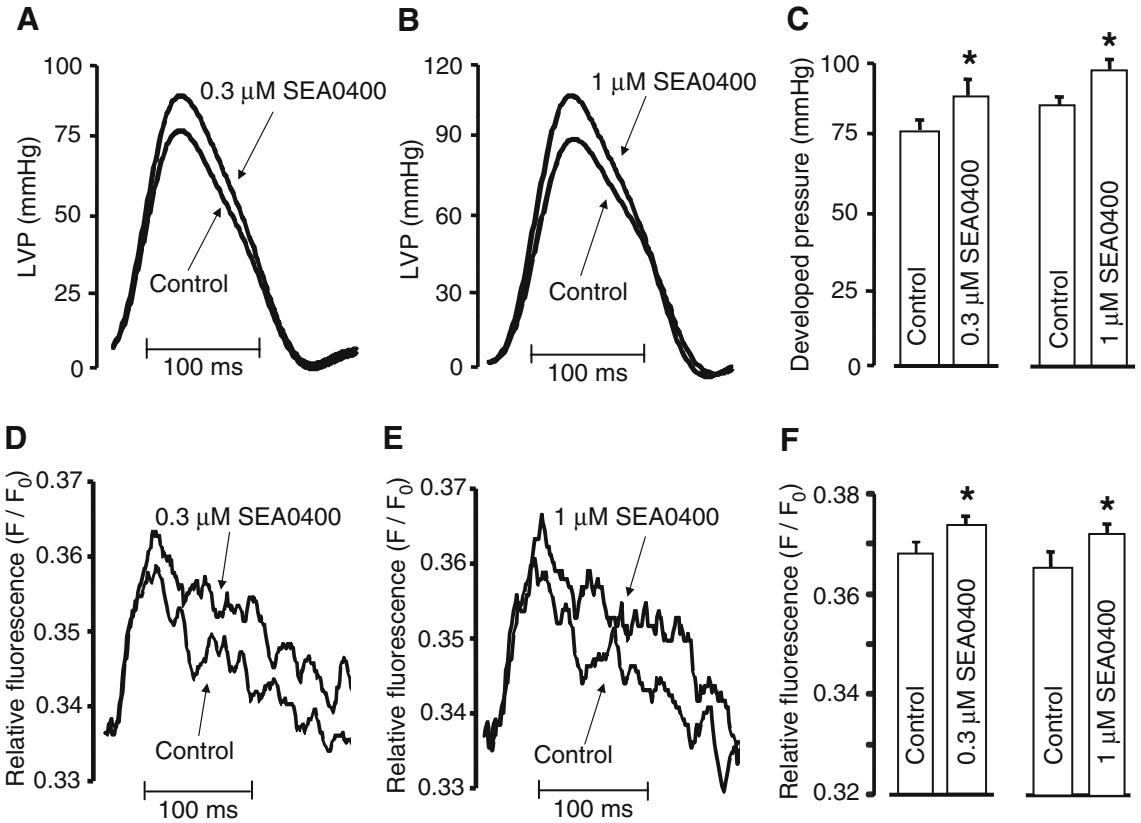

Fig. 4 Effect of SEA0400 on LVP and $[Ca^{2+}]_i$ in Langendorff-perfused rat hearts. Representative set of analogue records showing the changes in LVP (**a**, **b**) and $[Ca^{2+}]_i$ transients, displayed as changes in the fluorescent ratio of F/F_0, (**d**, **e**) before and after 20-min perfusion with 0.3 (**a**, **d**) and 1 μM (**b**, **e**) SEA0400. **c** Average values of developed pressure obtained in the absence and the presence of 0.3 (n=6) and 1 μM SEA0400 (n=8). **f** Respective average values obtained for the $[Ca^{2+}]_i$ amplitudes. *Columns* and *bars* represent mean values±SEM; *asterisks* indicate significant changes from control

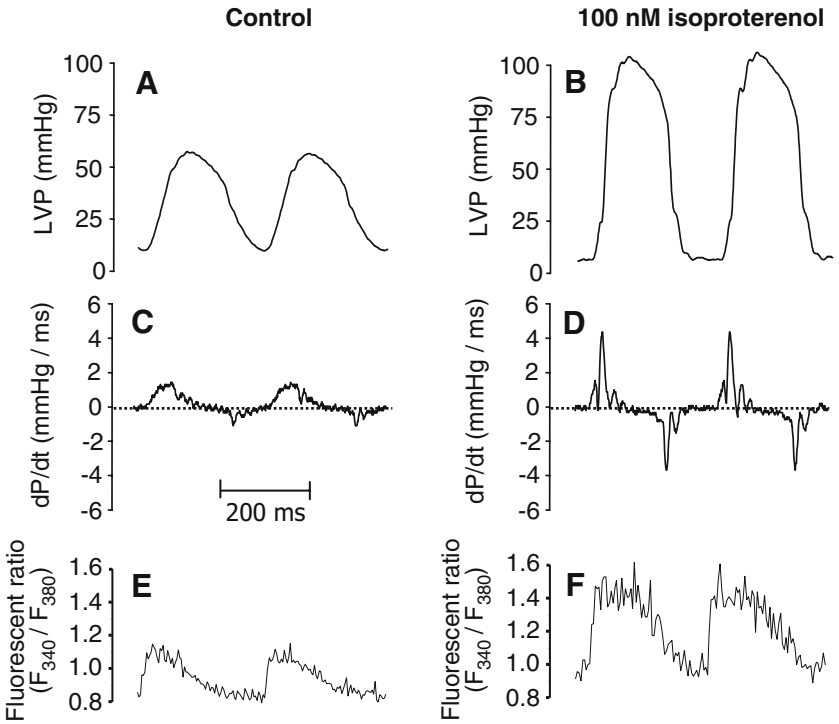

Fig. 5 Representative set of analogue records showing the changes in LVP (**a**, **b**), in their first time derivatives, dP/dt (**c**, **d**) and in $[Ca^{2+}]_i$ transients, (**e**, **f**) recorded from a Langendorff-perfused guinea pig heart before (**a**, **c**, **e**) and 1 min after (**b**, **d**, **f**) perfusion with 100 nM isoproterenol

species having a short action potential duration, like mice or rats, but not in other mammals likely including humans.

However, the fact that the suppression of NCX failed to elevate $[Ca^{2+}]_i$ in guinea pig myocardium requires further explanations. In the absence of a direct effect of SEA0400 on sarcoplasmic Ca^{2+} transport, this is only possible if the net effect of SEA0400 on transmembrane Ca^{2+} transport is close to zero, i.e., if the effects on the outward and inward Ca^{2+} fluxes are approximately equal. According to previous results, 1 μM SEA0400 blocked 80% of the inward NCX current in guinea pig and 60% of it in canine ventricular cells under patch-clamp conditions when $[Ca^{2+}]_i$ was kept at low levels (Tanaka et al. 2002; Birinyi et al. 2005). This extent of NCX inhibition is expected to cause $[Ca^{2+}]_i$ accumulation, resulting in increased $[Ca^{2+}]_i$ transients and contractility, if NCX is really the main mechanism of calcium extrusion from cardiac cells. Since such changes clearly failed to occur in the present experiments, one must assume that either the SEA0400-induced NCX inhibition is strongly reduced under physiological conditions (i.e., when $[Ca^{2+}]_i$ is not buffered) or SEA0400 may decrease calcium entry via L-type calcium channels as well. Indeed, the NCX-inhibitory effect of SEA0400 was found to be partially attenuated when $[Ca^{2+}]_i$ was increased (Lee et al. 2004; Bouchard et al. 2004), in accordance with our previous results obtained in canine ventricular cells indicating that the inhibitory effect of SEA0400 was halved when $[Ca^{2+}]_i$ was increased from 55 nM to 1 μM (Birinyi et al. 2008). Furthermore, 1 μM SEA0400 was shown to decrease the L-type calcium current in canine (Birinyi et al. 2005), rat (Acsai et al. 2007), and guinea pig (Tanaka et al. 2002) ventricular myocytes. Thus, in the case of SEA0400, both mechanisms are likely to be involved in balancing the reduced calcium extrusion.

At present, SEA0400 seems to be one of the most selective NCX blockers developed for therapeutic use, since it fails to modify ion currents in the surface membrane other than I_{Ca} (Tanaka et al. 2002). The combined suppression of $I_{Na/Ca}$ and I_{Ca} is not a unique feature of SEA0400 but has also been observed with several antiarrhythmic agents, like amiodarone (Watanabe and Kimura 2000), bepridil (Watanabe and Kimura 2001), or aprindine (Watanabe et al. 2002). Concomitant suppression of $I_{Na/Ca}$ and I_{Ca} may be therapeutically beneficial, since due to the parallel reduction of transmembrane Ca^{2+} influx and efflux, Ca^{2+} balance can be achieved at a lower cost of metabolic energy. Thus, SEA0400 is able to effectively decrease NCX activity under pathological conditions (i.e., during an ischemia/reperfusion episode) preventing thereby the proarrhythmic consequences of an ischemic injury (Takahashi et al. 2003) without leading to calcium overload in normal cardiac tissues.

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