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Src family protein tyrosine kinases modulate L-type calcium current in human atrial myocytes

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ABSTRACT

In the heart, L-type voltage dependent calcium channels (L-VDCC) provide Ca^{2+} for the activation of contractile apparatus. The best described pathway for L-type Ca^{2+} current ($I_{Ca,L}$) modulation is the phosphorylation of calcium channels by cAMP-dependent protein kinase A (PKA), the activity of which is predominantly regulated in opposite manner by β-adrenergic (β-ARs) and muscarinic receptors. The role of other kinases is controversial and often depends on tissues and species used in the studies. In different studies the inhibitors of tyrosine kinases have been shown either to stimulate or inhibit, or even have a biphasic effect on $I_{Ca,L}$. Moreover, there is no clear picture about the route of activation and the site of action of cardiac Src family nonreceptor tyrosine kinases (Src-nPTKs). In the present study we used PP1, a selective inhibitor of Src-nPTKs, alone and together with different activators of $I_{Ca,L}$, and demonstrated that in human atrial myocytes (HAMs): (i) Src-nPTKs are activated concomitantly with activation of cAMP-signaling cascade; (ii) Src-nPTKs attenuate PKA-dependent stimulation of $I_{Ca,L}$ by inhibiting PKA activity; (iii) $G\alpha_s$ are not involved in the direct activation of Src-nPTKs. In this way, Src-nPTKs may provide a protecting mechanism against myocardial overload under conditions of increased sympathetic activity.

1. Introduction

Src-nPTKs family are widely distributed in different tissues and play a role in many signaling pathways of different classes of cellular receptors that control a diverse spectrum of receptor-induced biological functions [1]. However, there are few reports about what type members of Src-nPTKs exist in the heart and what is their role in regulation of $I_{Ca,L}$ [2,3]. β-ARs mediate the positive inotropic effects of catecholamines by cAMP-dependent phosphorylation of the Ca^{2+} channels which provide Ca^{2+} for the initiation and regulation of cell contraction [4]. There have been reports of activating as well as depressing effects of Src-nPTKs inhibitors on $I_{Ca,L}$. For example, genistein, a nonselective inhibitor of PTKs, increased $I_{Ca,L}$ [5] or had biphasic effect on $I_{Ca,L}$ in HAMs [6] or feline [7] atrial myocytes, and reduced $I_{Ca,L}$ in guinea pig myocytes [8]. The stimulation of single L-VDCC activity by isoprenaline can be antagonized by phosphotyrosine phosphatase (PTP) inhibitors [9]. In addition, genistein enhanced the positive inotropic effect of norepinephrine induced through β-ARs in canine ventricular myocardium [10]. Interestingly, atrial fibrillation may be associated with impaired $I_{Ca,L}$ regulation by Src-nPTKs in HAMs [11]. It is of note that genistein may act nonselectively. For instance, there are reports that genistein inhibits $I_{Ca,L}$ [12,13] or inhibits the negative inotropic effect of endothelin-1 induced by crosstalk with norepinephrine on canine ventricular *trabeculae* [10] by a PTK-independent mechanism. These studies raise the question about the trustworthiness of Src-nPTKs inhibitors and do not provide a clear answer about the true role of Src-nPTKs in regulation of $I_{Ca,L}$.

It is not clear at which point of cAMP-dependent signaling cascade Src-nPTKs act and how they are activated. Src-nPTKs can be directly stimulated by $G\alpha_s$ and $G\alpha_i$ [14]. There are the reports that Src-nPTKs are activated by PKA phosphorylation at S17 residue [15,16]. Others have demonstrated that Src-nPTKs are activated by PKC but not by PKA and phosphatase 1 and 2A dependent pathways [17]. Also, there are evidences that Src-nPTKs can be activated directly by β_2-ARs or through β_2-AR-coupled $G\alpha_s$ proteins and/or PKA [18]. In the cAMP-dependent signaling cascade, β-ARs [19], Gα [20] and L-VDCC [21] have been demonstrated to be the possible targets of Src-nPTKs.

Abbreviations: L-VDCC, L-type voltage dependent calcium channels; $I_{Ca,L}$, L-type calcium current; PKA, protein kinase A; β-ARs, β-adrenergic receptors; Src-nPTKs, Src family nonreceptor protein tyrosine kinases; HAMs, human atrial myocytes; ISO, isoprenaline; AC, adenylyl cyclase.

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In the present study we demonstrate that in human atrial myocytes Src-nPTKs are activated concomitantly with activation of cAMP-dependent cascade and attenuate protein kinase A-dependent stimulation of $I_{Ca,L}$ through the negative feedback mechanism inhibiting PKA activity.

2. Materials and methods

2.1. Patients

Our investigations were performed in accordance with the principles outlined in the Declaration of Helsinki and approved by Kaunas Regional Bioethics Committee (BE-2-18, 2006). Specimens of right atrial *trabeculae* were obtained from 25 patients undergoing heart surgery for coronary artery diseases at the Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania). Most of the patients received a pharmacological pretreatment (Ca^{2+}-channel blockers, digitalis, β-AR antagonists, diuretics, ACE inhibitors, NO donors, and/or antiarrhythmic drugs) that was stopped 24 h before surgery. In addition, all patients received sedatives, anesthesia, and antibiotics. Dissociation of the cells was performed as described previously [22].

2.2. Solutions

The control external solution contained (in mM): NaCl 125; HEPES 10; CsCl 20; $NaHCO_3$ 4; NaH_2PO_4 0.8; $MgCl_2$ 1.8; $CaCl_2$ 1.8; D-glucose 5; sodium pyruvate 5; tetrodotoxin 5×10^{-4}; pH was 7.4. Patch electrodes (1–1.5 Mohms) were filled with control internal solution which contained (in mM): CsCl 138; EGTA 5; $MgCl_2$ 4; creatine phosphate disodium salt 5; Na_2ATP 3.1; Na_2GTP 0.42; HEPES 10; CaCl2 0.062 (pCa 8.5); pH = 7.3 was adjusted with CsOH. Collagenase type 2 was purchased from Worthington Biochemicals (NJ, USA). PP1 was from Alexis (CA, USA). All other drugs were from Sigma (Steinheim, Germany). Catalytic subunit of PKA was directly dissolved in the internal solution right before beginning of patch–clamp experiments, kept in the cold environment (+4 °C) and used within 24 h.

2.3. Whole-cell current recording

The whole-cell patch–clamp technique was used to record the $I_{Ca,L}$ in human atrial myocytes. In the routine protocols the cells were depolarized every 8 s from a holding potential of −80 mV by a short prepulse (50 ms) to −50 mV and then to 0 mV for 400 ms. The prepulse and the application of tetrodotoxin were used to eliminate fast sodium currents. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Voltage–clamp pulses were generated and currents recorded using a VP-500 patch–clamp amplifier (Bio-Logic, Claix, France). Control and drug containing solutions were applied to the exterior of the cell using a rapid solution changer (RSC-200, Bio-Logic, Claix, France). All experiments were performed at room temperature (19–25 °C), and the temperature did not vary by more than 1 °C in a given experiment.

2.4. Data analysis

The amplitude of whole-cell $I_{Ca,L}$ was measured as previously described [22]. The results are expressed as mean ± S.E.M. For statistical evaluation a paired Student's *t*-test was used, and a difference was considered significant when $p < 0.05$.

3. Results

3.1. Src-nPTKs attenuate PKA-dependent $I_{Ca,L}$ stimulation in HAMs

To examine the role of Src-nPTKs in the regulation of L-type calcium current, we measured basal $I_{Ca,L}$ before and after application of PP1 (Fig. 1A and C). PP1 (1 μM) had no effect on basal $I_{Ca,L}$ ($n = 8$). After washout of the compound, we used a nonsaturating concentration of isoprenaline, a nonselective agonist of β-ARs, to prestimulate $I_{Ca,L}$ and applied PP1 again (Fig. 1A). Isoprenaline (10 nM) alone stimulated $I_{Ca,L}$ by 291 ± 21% over control level. Then, PP1 applied in the continuous presence of isoprenaline additionally and reversibly stimulated $I_{Ca,L}$ by 22 ± 2.2% ($n = 5$; $p < 0.05$).

Thus, under control conditions, Src-nPTKs are inactive or have no effect on calcium channels in their nonphosphorylated state. The experiment shown in Fig. 1A suggests that Src-nPTKs attenuate PKA-dependent stimulation of $I_{Ca,L}$. To verify whether Src-nPTKs are active under control conditions or are activated concomitantly with activation of cAMP-dependent cascade, we examined the effect of PP1 after prestimulation of $I_{Ca,L}$ by lower concentrations of isoprenaline. 0.3 and 1 nM of isoprenaline stimulated $I_{Ca,L}$ by 30 ± 8.4% ($n = 6$) and 108 ± 11% ($n = 8$) over control, respectively. While after 0.3 nM of isoprenaline PP1 had no effect (1.2 ± 0.6%; $p > 0.05$), after 1 nM of isoprenaline it additionally stimulated $I_{Ca,L}$ by 12 ± 1.3% ($p < 0.01$). Fig. 1B summarizes the effects of PP1 after all used concentrations of isoprenaline. The experimental data were fitted with linear regression of the second order because at higher concentrations of isoprenaline the stimulation of $I_{Ca,L}$ approaches saturation. The effect of PP1 well correlated with the extent of $I_{Ca,L}$ prestimulation by isoprenaline ($r^2 = 0.83$). These experiments demonstrate that in control conditions Src-nPTKs are inactive and substantial activation of PKA is required to trigger their activity.

In further experiments we attempted to identify at which level of the cAMP-dependent cascade – including β-ARs, adenylyl cyclase (AC), PKA, phosphatases 1 and 2A, and L-VDCCs – the Src-nPTKs act (see Fig. 4). For this purpose, first we used nonsaturating concentration of forskolin, a direct activator of AC, assuming that if its effect would not be further potentiated by PP1 that would mean that Src-nPTKs act at the level of β-ARs. Otherwise Src-nPTKs act at the level of AC and PDEs, or down-stream from them.

As demonstrated in Fig. 1C, $I_{Ca,L}$ prestimulated by forskolin was further potentiated by PP1. Forskolin (0.03 μM) alone stimulated $I_{Ca,L}$ by 262 ± 11% above control level and PP1 reversibly potentiated this stimulation by 17 ± 3.2% ($n = 4$; $p < 0.05$), while it had no effect on basal $I_{Ca,L}$.

To verify whether Src-nPTKs regulate $I_{Ca,L}$ by modulating production of endogenous cAMP, we applied intracellular perfusion of cAMP to the myocyte, assuming that if its effect would not be further potentiated by PP1 that would mean that Src-nPTKs act at the level of AC and PDEs. Otherwise, Src-nPTKs act at the level of PKA, phosphatases or Ca^{2+} channels.

As shown in Fig. 2A, $I_{Ca,L}$ prestimulated by intracellularly applied cAMP was further potentiated by PP1. Since a low concentration of cAMP (300 μM) was directly added to the pipette, $I_{Ca,L}$ began to increase immediately after opening of the cell. When it reached maximum, application of PP1 (1 μM) potentiated the effect of cAMP by 24 ± 4.5% ($n = 5$; $p < 0.05$).

This experiment suggests that Src-nPTKs act at the end of cAMP-dependent signaling cascade, i.e., at the level of L-VDCCs. Possible actions of Src-nPTKs at this level could be: attenuation of PKA-stimulated Ca^{2+} channel activity; inhibition of PKA catalytic activity; stimulation of phosphatase activity. In the heart, the major phosphatases are type 1 and 2A, which together comprise more than 90% of the phosphatases in cardiac myocytes

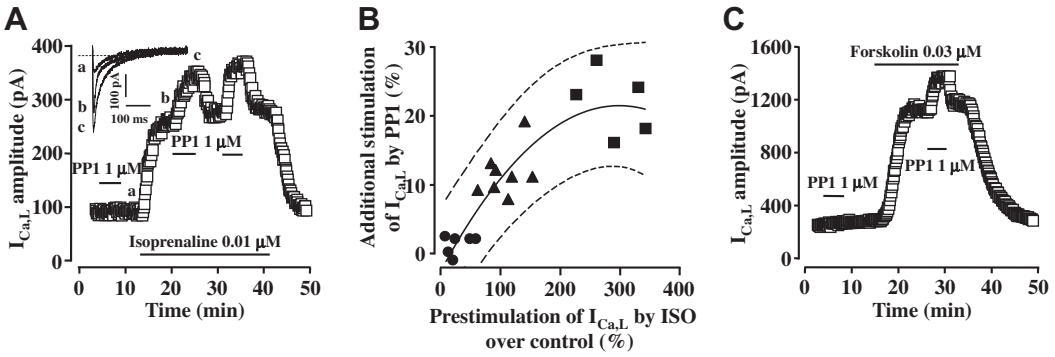

Fig. 1. Effect of PP1 on basal and isoprenaline (A) and forskolin (C) stimulated $I_{Ca,L}$ in human atrial myocytes. Each square represents the peak amplitude of $I_{Ca,L}$ measured every 8 s. During the periods indicated by the horizontal lines, the cell was exposed to the indicated compound. The current traces shown on the top of the graph A were recorded at the times indicated by corresponding letters in the graph. (B) The dependence of the effect of PP1 on the extent of $I_{Ca,L}$ prestimulation. The effects 0.3, 01 and 10 nM of isoprenaline (ISO) are represented by circles, triangles and squares, respectively. All points were fitted using linear regression of the second order (solid line). Correlation coefficient r^2 was 0.83. Dotted lines indicate the prediction intervals.

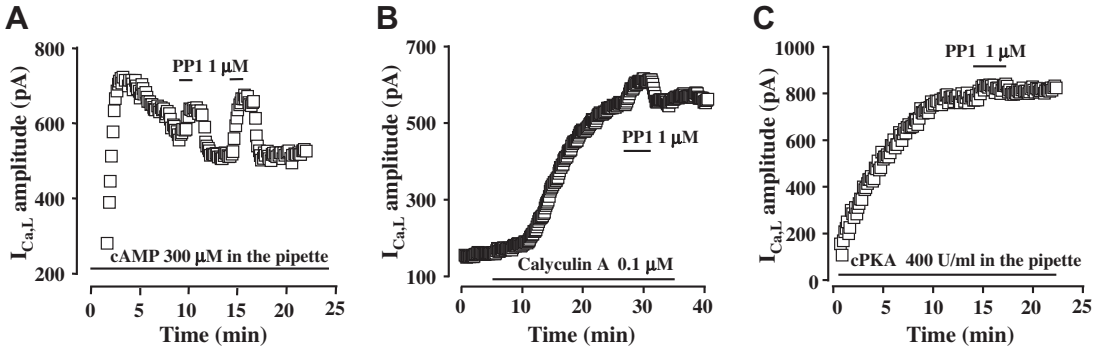

Fig. 2. Effect of PP1 on intracellular cAMP (A), calyculin A (B) and catalytic subunit of PKA (C) stimulated $I_{Ca,L}$ in human atrial myocytes. Each symbol represents the peak amplitude of $I_{Ca,L}$ measured every 8 s. During the periods indicated by the horizontal lines, the cell was exposed to the indicated compound.

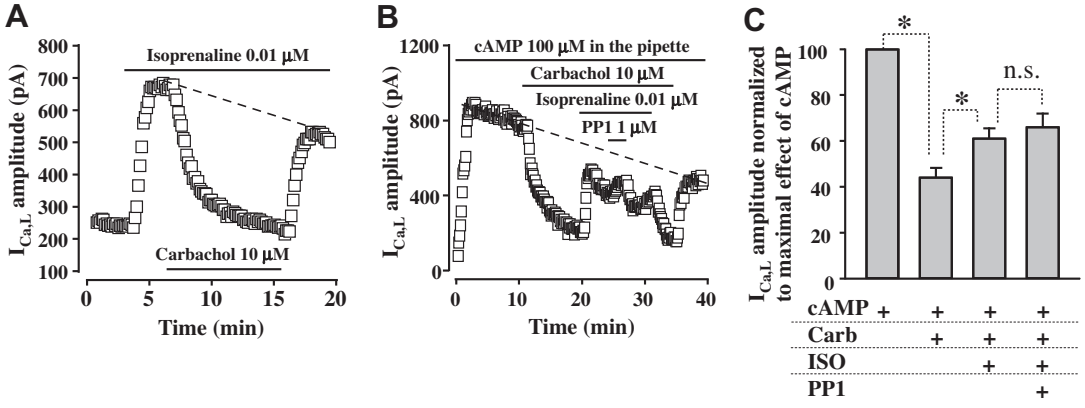

Fig. 3. $G\alpha_s$ proteins are not involved in the activation of Src-nPTKs in human atrial myocytes. (A) The effect of isoprenaline (10 nM) on $I_{Ca,L}$ can be completely prevented by AC inhibitor carbachol (10 μM). (B) $I_{Ca,L}$ was prestimulated by cAMP, which was intracellularly applied through the patch pipette. Then the activity of AC was blocked by carbachol which partially inhibited $I_{Ca,L}$. Under such conditions, isoprenaline applied to stimulate $G\alpha_s$ had no inhibitory effect on $I_{Ca,L}$. In the continuous presence of all mentioned compounds, PP1 slightly stimulated $I_{Ca,L}$. (C) Summary of the effect of cAMP alone and together with carbachol (Carb), isoprenaline (ISO) and PP1. *-$p < 0.05$; n.s.-not significant.

[23]. Another, Ca^{2+}-dependent cardiac phosphatase 2B (calcineurin), is not supposed to play a role in the whole-cell patch–clamp mode where EGTA is used to chellate intracellular Ca^{2+} ions. There is no data in the literature that phosphatases 1 and 2A can be activated by tyrosine phosphorylation. On the contrary, tyrosine phosphorylation inactivates phosphatase 1 [24] and phosphatase 2A [25]. However, indirectly Src-nPTKs might regulate phosphatase activity by phosphorylation of phosphatase 1 inhibitor I-1 and phosphatase 2A inhibitors I-1^{PP2A} and I-2^{PP2A} or DARPP-32 [26]. Therefore, to examine whether Src-nPTKs regulate $I_{Ca,L}$ by interfering with phosphatase activating pathways, we used calyculin A, a cell permeant inhibitor of phosphatases 1 and 2A. We assumed that a high concentration of calyculin A (0.1 μM) would completely block phosphatase 1 and 2A activities

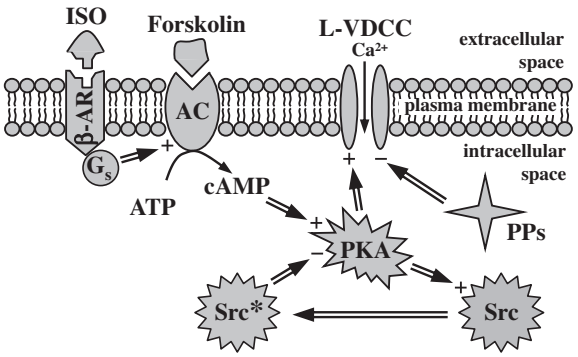

Fig. 4. Role of Src-nPTKs in regulation of L-VDCC activity. PKA, activated by cAMP-dependent signaling, stimulates L-VDCCs and concomitantly activates Src-nPTKs which under control conditions are inactive (Src). Once activated, Src-nPTKs (Src*) inhibit by the negative feedback mechanism the activity of PKA and, consequently, attenuate PKA-dependent stimulation of L-VDCCs. The main elements of the cAMP-dependent cascade are β-ARs, Gs proteins, AC, PKA, phosphatases 1 and 2A (PPs) and L-VDCC.

and induce stimulation of $I_{Ca,L}$ due to some basal PKA activity. Then, if PP1 will additionally stimulate $I_{Ca,L}$, that would indicate that Src-nPTKs do not change the phosphatase activity.

As demonstrated in Fig. 2B, calyculin A-prestimulated $I_{Ca,L}$ was further potentiated by PP1. Calyculin A (0.1 μM) alone stimulated $I_{Ca,L}$ by 224 ± 10% above control level and PP1 reversibly potentiated this stimulation by 8.6 ± 1.6% ($n = 6$; $p < 0.05$). This potentiation was lower than in the cases of isoprenaline, forskolin and cAMP, consistently with lower activity of cAMP-dependent PKA, which in the case of calyculin A remained at the basal level.

In the next series of experiments we examined two more possible ways for Src-nPTKs-dependent $I_{Ca,L}$ depression, i.e., that Src-nPTKs act by inhibiting PKA activity or by inhibiting PKA-stimulated L-VDCC activity. Through the patch pipette, we applied the catalytic subunit of PKA (cPKA), the activity of which cannot be modulated through its regulatory subunits since they are absent, assuming that if its effect would not be further potentiated by PP1 that would mean that Src-nPTKs act by inhibiting PKA activity. Otherwise, Src-nPTKs directly inhibit PKA-stimulated Ca^{2+} channel activity.

As shown in Fig. 2C, intracellular perfusion with cPKA (400 U/ml) induced rapid increase in $I_{Ca,L}$ amplitude. The actual extent of $I_{Ca,L}$ stimulation by cPKA could not be measured precisely because after initiation of patch–clamp conditions $I_{Ca,L}$ usually is subject to some transient run-down, which in our case coincided with immediate stimulation by cPKA. Measuring from the first recorded current after cell rupture, we assumed that stimulation of $I_{Ca,L}$ by cPKA (400 U/ml) was more than 220% above control ($n = 5$). When $I_{Ca,L}$ reached a steady-state, application of PP1 (1 μM) did not further potentiate the effect of cPKA (2.9 ± 2.2%; $n = 5$; $p > 0.05$).

3.2. Are $G\alpha_s$ involved in the direct activation of Src-nPTKs?

It has been shown before that Src-nPTKs may be activated directly by β_2-ARs or by β_2-AR-coupled $G\alpha_s$ proteins, and/or PKA [18]. Our experiments showed that cAMP-dependent PKA activity was required for Src-nPTKs manifestation; however, we could not reject the additional route of Src-nPTKs activation through $G\alpha_s$. To verify this possibility, at first we demonstrated that stimulation of $I_{Ca,L}$ by 0.01 μM of isoprenaline could be completely blocked by 10 μM of carbachol, an inhibitor of AC (Fig. 3A; $n = 5$).

Then we used the following protocol (Fig. 3B): myocyte was internally perfused with cAMP (300 μM) to prestimulate $I_{Ca,L}$ and activate Src-nPTKs. When a steady-state of $I_{Ca,L}$ stimulation was reached, a high concentration of carbachol (10 μM) was added to the external solution to completely block AC activity and prevent its stimulation by $G\alpha_s$. Then isoprenaline (0.01 μM) was applied to stimulate $G\alpha_s$. Assuming that if Src-nPTKs are activated by $G\alpha_s$, in the presence of carbachol, isoprenaline should attenuate cAMP-induced stimulation of $I_{Ca,L}$. However, in such conditions, isoprenaline had no inhibition and, in contrast, exerted quite substantial stimulation of $I_{Ca,L}$ suggesting that $G\alpha_s$ may be involved not in direct activation but in direct inhibition of Src-nPTKs, because in the continuous presence of isoprenaline PP1 had only a marginal additional stimulatory effect on $I_{Ca,L}$. The summary of this experiment is presented in Fig. 3C. Since the exact basal $I_{Ca,L}$ could not be measured, $I_{Ca,L}$ was normalized to the maximal effect of cAMP. Successive wash in of carbachol, isoprenaline and PP1 to the perfusing solution resulted in $I_{Ca,L}$ amplitude equal to 44 ± 4.2%, 61 ± 4.9% and 66 ± 6.3% ($n = 6$) of the cAMP effect, respectively. Interestingly, carbachol as an AC inhibitor was not supposed to inhibit the effect of intracellularly applied cAMP on $I_{Ca,L}$. This unexpected inhibitory effect could be reached due to the reduction of cAMP concentration within a compartment near L-type Ca^{2+} channel by cGMP-stimulated PDE2 [27].

4. Discussion

4.1. The mode of Src-nPTKs activation and action

Our experiments demonstrate that Src-nPTKs have no effect on basal $I_{Ca,L}$ but attenuate PKA-dependent stimulation of $I_{Ca,L}$. Under control conditions, Src-nPTKs are repressed consistently with an idea that in normal cells Src-nPTKs should be kept in an inactive state due to their oncogenic properties [14,28,29] and PKA activity may be required for Src-nPTKs activation [18]. Some stimulatory effect of PKA and inhibitory effect of Src-nPTKs on $I_{Ca,L}$ could be concealed by highly active phosphatases. However, in our experiments higher threshold concentrations of isoprenaline were required to trigger Src-nPTKs than to induce $I_{Ca,L}$ stimulation pointing to that under control conditions Src-nPTKs are inactive. Even under conditions when PKA was sufficiently acivated by the lowest used concentration of isoprenaline (0.3 nM) to initiate stimulation of $I_{Ca,L}$, administration of PP1 still did not reveal any activity of Src-nPTKs. Fig. 1B suggests that Src-nPTKs were triggered only at higher concentrations of isoprenaline and then the activities of both kinases increased concomitantly since the larger effect of PP1 on $I_{Ca,L}$ was observed when stimulation of $I_{Ca,L}$ was stronger. Accordingly, when $I_{Ca,L}$ was prestimulated by phosphatase inhibitor calyculin A (Fig. 2B), which did not change the basal activity of PKA, the PP1 exerted a relatively small additional stimulatory effect on $I_{Ca,L}$ affirming that the activation of Src-nPTKs is PKA-dependent.

4.2. The site of Src-nPTKs action

It has been demonstrated that Src-nPTKs activated by α1-ARs in guinea pig ventricular myocytes have inhibitory effect on β-adrenergic responses at the level of the β-ARs [19]. Other studies show that Src-nPTKs may act on β-ARs as a desensitizing factor, associated with "run-down" after agonistic stimulation of β-ARs [30,31], or that Src-nPTKs can phosphorylate and thereby increase activity of Gα subunits [20,32]. Our experiments, in which PP1 potentiated $I_{Ca,L}$ prestimulated by signaling proteins consecutively going down β-adrenergic cascade, indicate that Src-nPTKs act remotely from β-ARs, at the level of L-VDCCs. Both kinases, PKA and Src-nPTKs, have phosphorylation sites on the cardiac calcium channels. PKA phosphorylates S1928 of channel pore forming subunit α1 and S478/479 on regulatory β subunit [33], while Src-nPTKs have been shown to bind to the II–III linker and C-terminal

domain of α1 subunit [21]. However, our experiments in which we used catalytic subunit of PKA to stimulate $I_{Ca,L}$ show that Src-nPTKs regulate the activity of PKA rather than the activity of L-VDCCs (Fig. 4).

Human regulatory subunits of PKA have multiple tyrosine residues as the possible targets of Src-nPTKs phosphorylation (GenBank ID: NP_997636.1, NP_001158232.1, NP_004148.1, NP_002727.2). Src-nPTKs prefer EEIYGEF as substrate [34] which is not present in the regulatory subunits of PKA; however, this preference is not very stringent, and Src-nPTKs can tolerate amino acid substitutions without drastic consequences. Anyway, there is no data in the literature on PTK-dependent regulation of PKA activity, while in general, phosphorylation can change the affinity of PKA regulatory subunit to its catalytic subunit as it has been shown in *in vitro* studies with autophosphorylation of human PKA type 2 regulatory subunit [35]. Such a negative feedback mechanism when elevated PKA activity is required for triggering the Src-nPTKs, which, in turn, diminish the activity of PKA by phosphorylation of tyrosine residues of its regulatory subunits, may serve as a protector from myocardial overload under conditions of increased sympathetic activity.

4.3. The route of Src-nPTKs activation in human atrial myocytes

McGarrigle provides a scheme depicting three possible routes of Src-nPTKs activation involving β-ARs, trimeric G_s proteins, AC and PKA [18]. The first and the second routes come into play at a low (<100 nM) concentration of isoprenaline, when Src-nPTKs are activated directly by $G\alpha_s$ or by $G\alpha_s$-AC-cAMP-activated PKA. The third route is initiated at a high (>100 nM) concentration of isoprenaline, when Src-nPTKs are additionally activated directly through β_2-ARs. Since in our experiments we used 10 nM of isoprenaline, the first and the second routes could be involved in the activation of Src-nPTKs. The experiment presented in Fig. 3B indicates that $G\alpha_s$ have no stimulatory effect on Src-nPTKs; on the contrary, under conditions of completely inhibited AC, isoprenaline induced additional stimulation of cAMP-prestimulated $I_{Ca,L}$ suggesting that $G\alpha_s$ could inhibit Src-nPTKs because in the continuous presence of isoprenaline PP1 had only a marginal additional stimulatory effect on $I_{Ca,L}$.

In summary, our findings demonstrate that in human atrial myocytes: (i) Src-nPTKs are activated concomitantly with activation of cAMP-signaling cascade; (ii) Src-nPTKs attenuate PKA-dependent stimulation of $I_{Ca,L}$ by inhibiting PKA activity; (iii) $G\alpha_s$ are not involved in the direct activation of Src-nPTKs. In this way, Src-nPTKs may provide a protecting mechanism against myocardial overload under conditions of increased sympathetic activity.

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