J Physiol Sci (2016) 66:327–336
DOI 10.1007/s12576-015-0430-3

ORIGINAL PAPER

Differences in the control of basal L-type Ca^{2+} current by the cyclic AMP signaling cascade in frog, rat, and human cardiac myocytes

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Received: 30 July 2015 / Accepted: 26 November 2015 / Published online: 16 December 2015

Abstract β-adrenergic receptors (β-ARs) mediate the positive inotropic effects of catecholamines by cAMP-dependent phosphorylation of the L-type Ca^{2+} channels (LTCCs), which provide Ca^{2+} for the initiation and regulation of cell contraction. The overall effect of cAMP-modulating agents on cardiac calcium current ($I_{Ca,L}$) and contraction depends on the basal activity of LTCCs which, in turn, depends on the basal activities of key enzymes involved in the cAMP signaling cascade. Our current work is a comparative study demonstrating the differences in the basal activities of β-ARs, adenylyl cyclase, phosphodiesterases, phosphatases, and LTCCs in the frog and rat ventricular and human atrial myocytes. The main conclusion is that the basal $I_{Ca,L}$, and consequently the contractile function of the heart, is secured from unnecessary elevation of its activity and energy consumption at the several "checking-points" of cAMP-dependent signaling cascade and the loading of these "checking-points" may vary in different species and tissues.

Keywords Cardiac myocytes · L-type Ca^{2+} channels · Adenylyl cyclase · Phosphodiesterase · Phosphatase · Protein kinase A

Introduction

The cardiac L-type calcium current ($I_{Ca,L}$) plays a determinant role in the initiation and development of cardiac contraction [1, 2]. It is therefore crucial to understand how its amplitude can be regulated. One of the most common pathways for the modulation of $I_{Ca,L}$ amplitude is the β-adrenergic receptor (β-ARs)/cAMP signaling cascade resulting in the phosphorylation of L-type voltage-dependent Ca^{2+} channels (LTCCs) by the cAMP-dependent protein kinase A (PKA). The major elements of the β-AR/cAMP signaling cascade are the stimulatory G_s proteins, adenylyl cyclase (AC), cAMP, phosphodiesterases (PDEs), PKA, and protein phosphatases (PPs) [1]. The overall effect of cAMP-modulating agents on cardiac $I_{Ca,L}$ and contraction depends on the basal activity of LTCCs which, in turn, depends on the basal activities of key enzymes involved in the cAMP signaling cascade. For instance, activators of AC, such as β-adrenergic agonists or forskolin, or inhibitors of PDE activity, such as 1-methyl 3-isobutyl xanthine (IBMX), lead to an increase in intracellular cAMP concentration ($[cAMP]_i$) and to stimulation of $I_{Ca,L}$ due to phosphorylation of LTCCs, the extent of which depends on PKA and PPase balance. Conversely, agents that inhibit or suspend AC activity, such as muscarinic agonists or inverse agonists of β-ARs, reduce $[cAMP]_i$ and inhibit $I_{Ca,L}$. Basal level of $[cAMP]_i$ within the myocyte, and in particular within the intracellular compartments that regulate LTCC phosphorylation, determines a "basal" activity of LTCCs and a basal $I_{Ca,L}$ amplitude [3–5].

In the present study, we explored whether basal AC, PDE, PKA, and PPase activities control the basal amplitude of $I_{Ca,L}$ in cardiac myocytes freshly isolated from adult frog (FVM) and rat (RVM) ventricles and from human atria (HAM). For that, we compared the effects of acetylcholine (ACh), IBMX, PKI(15-22), and calyculin A (Cal A) on basal $I_{Ca,L}$ amplitude.

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Methods

Our investigation conforms to the European Community guidelines for the care and use of animals (86/609/CEE, CE Off J no. L358, December 18, 1986). The license for the use of laboratory animals (No. 0122, 12-12-2004) was received from the Lithuanian Food and Veterinary Service. Protocols for obtaining human cardiac tissues were approved by Kaunas Regional Bioethics Committee, Lithuania (No. BE-12-18, 2006).

Frog ventricular myocytes

Hearts from frog (*Rana esculenta*) were enzymatically dispersed by a combination of collagenase (Yakult) and trypsin (type XIII, Sigma) as described [6]. Frogs were killed by decapitation, the spinal cord was destroyed with a steel rod, and the heart was then excised. The isolated cells were stored in storage Ringer solution, and kept at 4 °C.

Rat ventricular myocytes

Adult rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (160–180 g) as previously described [7]. The myocytes were maintained at 37 °C until use.

Human atrial myocytes

Specimens of right atrial *trabeculae* were obtained from 15 patients (11 males and four females) undergoing heart surgery for congenital defects, coronary artery diseases, valve replacement, or heart transplantation at the Department of Cardiothoracic and Vascular Surgery of the Hospital of Lithuanian University of Health Sciences, Kaunas, Lithuania. Most 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 immediately after surgery as described previously [8]. The cell suspension was filtered, centrifuged, and the pellet resuspended in Dulbecco's minimal essential medium supplemented with 10 % fetal calf serum, nonessential amino acids, 1 nM insulin, and antibiotics (100 IU/ml penicillin and 0.1 μg/ml streptomycin).

Solutions

For electrophysiology, the control external solution contained (in mM): NaCl 107 for frog or 125 for rat and human; 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 3×10^{-4} (for frog) or 6×10^{-3} (for rat and human); pH 7.4 adjusted with NaOH. Patch electrodes (0.6–1.5 MOhms) were filled with control internal solution which contained (mM): CsCl 120 for frog or 138 for rat and human; EGTA (acid form) 5; $MgCl_2$ 4; creatine phosphate disodium salt 5; Na_2ATP 3.1; Na_2GTP 0.42; $CaCl_2$ 0.062 (pCa 8.5); HEPES 10; pH 7.1 (frog) or 7.3 (rat and human) adjusted with CsOH. Collagenase type 2 was purchased from Worthington Biochemical Corporation. Tetrodotoxin was from Latoxan; Cal A—from Gibco; H89—from Calbiochem. All other drugs were from Sigma-Aldrich.

Whole-cell current recording

The whole cell configuration of the patch-clamp technique was used to record the high-threshold calcium current of Ca^{2+}-tolerant cardiac myocytes. In the routine protocols, the cells were depolarized every 8 s from a holding potential of −80 to 0 mV for 200 or 400 ms. In HAMs and RVMs, the test pulse to 0 mV was preceded by a short prepulse to −50 mV. The prepulse and/or the application of TTX 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). Visual-Patch v1.30 computer software was used to control all experimental parameters, cell stimulation, and current recording. Control and drug-containing solutions were applied to the exterior of the cell by placing the cell at the opening of 300-μm-inner-diameter capillary tubes that flow at a rate of about 50 μl/min. Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC-200; Bio-Logic). All experiments were done at room temperature (19–25 °C), and the temperature did not vary by more than 1 °C in a given experiment.

Internal perfusion of myocytes

PKI(15-22), a peptide inhibitor of PKA, was delivered to the interior of the cell using a pipette holder that permitted the solution inside the patch pipette to be changed during an experiment [9, 10]. A thin capillary, pulled from 0.3 mm i.d., 0.7 mm o.d., polyethylene catheter, was inserted into the patch pipette to ∼0.5 mm of the tip. The distal end of the capillary was placed into reservoirs with control or PKI(15-22)-containing solutions. The pipette solution was changed by applying negative pressure to the pipette, which aspirated the solution from the capillary with flow rate of ∼5 μl/min. The negative pressure was

maintained throughout the experiment except while moving the capillary between reservoirs. We used pipettes with resistances ~1 MOhm, and the time to onset of the PKI(15-22) effect was 2–3 min.

Statistical analysis

The maximal amplitude of whole-cell $I_{Ca,L}$ was measured as previously described [6]. Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was done for each membrane depolarization to determine peak and steady-state current values. The results are expressed as mean ± SEM. For statistical evaluation, Student's *t* test was used, and a difference was considered statistically significant when p was <0.05.

Results

Control of basal $I_{Ca,L}$ by the cAMP-dependent cascade in FVMs

In FVMs, only β_2-ARs are functionally coupled to $I_{Ca,L}$ [11], and stimulation of β_2-ARs produces a strong increase in $I_{Ca,L}$ exclusively due to cAMP-PKA-dependent phosphorylation [10]. A total efficacy of β-AR stimulation of $I_{Ca,L}$ depends on the status of $I_{Ca,L}$ in control conditions, i.e., whether it is already preactivated by PKA, the activity of which in turn depends on basal activity of other enzymes of the signaling cascade. To verify the basal activity of AC, we used acetylcholine (ACh), a muscarinic receptor agonist that inhibits AC activity via the inhibitory G_i protein. ACh had no effect on basal $I_{Ca,L}$ in FVMs ($n = 6$), although it produced a potent and concentration-dependent inhibition of isoprenaline (ISO) stimulated $I_{Ca,L}$ (Fig. 1a). The stimulatory effect of ISO (1 μM) in different series of experiments ranged from 4 to 9 times stimulation of $I_{Ca,L}$ over basal amplitude. ACh completely blocked the effect of ISO (4 ± 11 % over basal; $n = 4$; $p < 0.005$; Fig. 1b). The effect of ACh was mimicked by PKI(15-22), a peptide inhibitor of PKA (Fig. 1c). When internally perfused into the myocyte, PKI(15-22) (20 μM) completely reversed ISO-stimulated $I_{Ca,L}$ to its basal level (Fig. 1d) (7 ± 8 % over basal; $n = 4$; $p < 0.005$). Thus, under basal conditions, AC is either inactive or its activity is overcome by the activity of PDEs and/or PPases. To distinguish between these two hypotheses, we first used IBMX, a wide-spectrum nonselective PDE inhibitor. IBMX alone produced a small but not significant stimulatory effect on basal $I_{Ca,L}$ (10 ± 7 % over basal; $n = 6$; Fig. 1e, f). However, when $I_{Ca,L}$ was initially prestimulated with a low concentration of ISO (5 nM; 73 ± 22 % over basal; $n = 4$; $p < 0.05$), IBMX further increased $I_{Ca,L}$ to a maximal stimulation of 762 ± 91 % above basal ($n = 4$; $p < 0.01$; Fig. 1f). This suggests that, under basal conditions, the activity of both AC and PDEs is very low. Upon a submaximal stimulation of the β-ARs, AC is turned on, cAMP is generated, and PDE activity now limits the rise in cAMP concentration and the activation of the cAMP/PKA cascade around LTCCs.

Next, we used calyculin A (Cal A), an inhibitor of PPase1 and PPase2A. In contrast to IBMX, Cal A (100 nM) induced a nearly maximal stimulation of basal $I_{Ca,L}$ (665 ± 181 % over basal; $n = 6$; $p < 0.005$; Fig. 2a, d). This suggests that PKA phosphorylation of LTCCs may occur at basal conditions but is too low to increase $I_{Ca,L}$ because it is counter-balanced by a high PPase activity. Surprisingly, the stimulatory effect of Cal A on basal $I_{Ca,L}$ remained unchanged even when the cells were pre-incubated during 10 min in 10 μM ACh to block basal AC activity (635 ± 172 % over basal; $n = 4$; $p < 0.05$; Fig. 2b) suggesting that ACh is incapable to completely inhibit cAMP synthesis, and even extremely low concentrations of cAMP may be sufficient for substantial activation of PKA. Yet, a balance between cAMP-dependent PKA phosphorylation and dephosphorylation of LTCCs must exist at basal because when Cal A was used at a subthreshold concentration (1 nM), which had no effect on basal $I_{Ca,L}$ per se, IBMX (which also had no effect per se as shown above, Fig. 1) now produced a strong stimulation of $I_{Ca,L}$ (570 ± 122 % over basal; $n = 4$; $p < 0.01$; Fig. 2c, d). Altogether, these experiments indicate that in FVMs basal AC and PKA activity is strongly balanced by PDE and PPase activity, respectively, so that basal $I_{Ca,L}$ is carried by LTCCs that are not phosphorylated by PKA.

Control of basal $I_{Ca,L}$ by the cAMP-dependent cascade in RVMs

Similar experiments were performed in RVMs. Unlike in FVMs, β-AR stimulation in RVMs is due to both β_1- and β_2-ARs, with a relative dominance of β_1-ARs [12]. Both receptors are coupled to AC and increase $I_{Ca,L}$ via PKA-dependent phosphorylation [10, 13]. Like in FVMs, ACh had no effect on basal $I_{Ca,L}$ amplitude in RVMs (−9 ± 14 %; $n = 5$; Fig. 3a). This indicates that basal AC activity is insufficient to stimulate LTCCs in RVMs. In contrast to FVMs, in RVMs ACh only partially inhibited the $I_{Ca,L}$ response to ISO (−17 ± 5 %; $n = 6$; $p < 0.05$; Fig. 3a, c) [14]. As shown earlier [15], the effect of ISO was completely blocked by 20 μM PKI(15-22) confirming that ISO-stimulation of $I_{Ca,L}$ was entirely due to PKA-dependent phosphorylation (Fig. 3b, c; $n = 4$; $p < 0.01$).

Unlike in FMVs, IBMX produced a strong stimulation of basal $I_{Ca,L}$ in RVMs (165 ± 21 % over basal; $n = 6$;

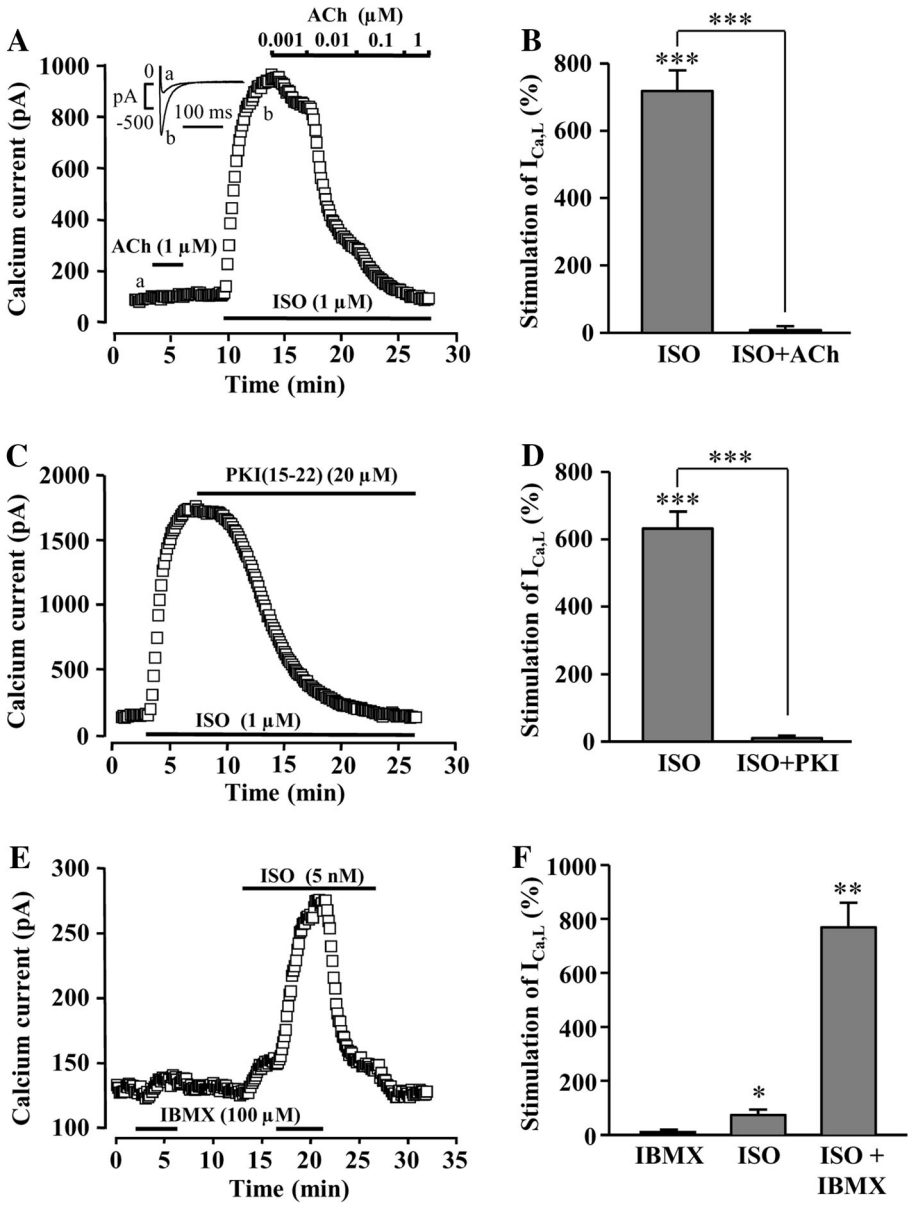

Fig. 1 Control of basal $I_{Ca,L}$ by β-AR-PKA signaling cascade in FVMs. **a** During the periods indicated by the *horizontal lines*, the cell was exposed to ACh or/and ISO. *Each symbol* represents the peak amplitude of $I_{Ca,L}$ obtained by depolarizing the cell every 8 s–0 mV over a period of 200 ms from a holding potential of −80 mV. The current traces shown in the inset were recorded at the times indicated by the *corresponding letters on the main graphs*. **b** *Bar graphs* representing the average effect of ISO, alone and together with ACh, on $I_{Ca,L}$. **c** After stimulation of $I_{Ca,L}$ with ISO, the intracellular solution was changed to one containing 20 μM PKI(15-22), which perfused the cell throughout the rest of experiment. **d** *Bar graphs* representing the average effect of ISO, alone and together with PKI(15-22), on $I_{Ca,L}$. **e** IBMX (100 μM) alone has no effect but remarkably potentiates the ISO-preactivated $I_{Ca,L}$. **f** *Bar graphs* representing the average effects of IBMX and ISO, alone and together, on $I_{Ca,L}$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, compared with basal $I_{Ca,L}$ or between indicated groups

$p < 0.01$; Fig. 3d, f), which was comparable to the maximal effect of ISO since the addition of either ISO or forskolin on top of IBMX had no further effect (not shown). This may indicate a larger basal AC activity in RVMs as compared to FVMs. To examine the role of PPase activity, RVMs were exposed to 100 nM Cal A. Like in FVMs, Cal A produced a strong increase in basal $I_{Ca,L}$ (Fig. 3e; 167 ± 62 %; $n = 4$), and the effect could be further slightly enhanced by adding ISO (1 μM; 21 ± 7 % on top of Cal A; $n = 4$; $p < 0.01$; Fig. 3e, f). Altogether, these experiments indicate that, similarly to FVMs, basal AC and PKA activity in RVMs is strongly balanced by PDE and PPase activity, so that basal $I_{Ca,L}$ is carried by LTCCs which are essentially not phosphorylated by PKA. However, basal AC activity is higher in RVMs than in FVMs, allowing the counterweight produced by PDE on LTCC phosphorylation to be easily removed in RVMs by PDE inhibition.

Control of basal $I_{Ca,L}$ by the cAMP-dependent cascade in HAMs

In the last series of experiments, we examined the control of basal $I_{Ca,L}$ by the cAMP/PKA cascade in HAMs. As shown earlier, β-AR stimulation of $I_{Ca,L}$ in HAMs is due to β_1-, β_2- and β_3-ARs which all produce a PKA-dependent activation of the current [10, 16]. In contrast to FVMs and RVMs, exposure of HAMs to ACh (10 μM) produced a

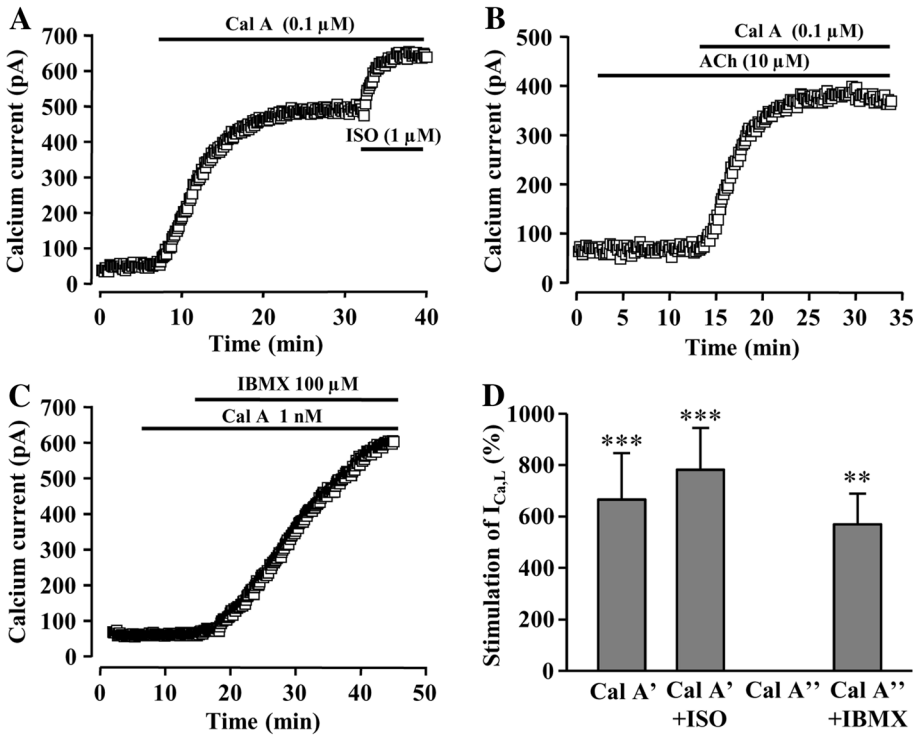

Fig. 2 The phosphatase inhibitor calyculin A near maximally stimulates basal $I_{Ca,L}$ in FVMs. **a** The cell was exposed to high concentration of Cal A (Cal A', 100 nM) and then to ISO (1 μM). **b** The myocyte was superfused with ACh (10 μM) to completely inhibit AC activity and then to high concentration of Cal A as in **a**. **c** The myocyte was superfused with low concentration of Cal A (Cal A″, 1 nM) and then to IBMX (100 μM). **d** *Bar graphs* representing the average effects of Cal A, alone and together with ISO or IBMX, on $I_{Ca,L}$. **p < 0.01; ***p < 0.005, compared with basal $I_{Ca,L}$

clear inhibition of basal $I_{Ca,L}$ (22 ± 8 % below basal; $n = 6$; $p < 0.01$; Fig. 4a, g). The effect was maximal, because increasing the concentration of ACh to 100 μM had no additional effect (not shown). However, the inhibitory effect of ACh on basal $I_{Ca,L}$ was observed only in 7 out of 11 patients (~64 %), without any clear correlation with the patients' clinical data. Addition of ACh on top of a saturating concentration of ISO (1 μM), which by itself increased $I_{Ca,L}$ >twofold (245 ± 32 % of basal; $n = 4$; $p < 0.005$; Fig. 4g), completely abolished the ISO effect (Fig. 4a, g). Moreover, $I_{Ca,L}$ amplitude in ISO + ACh was below basal level (29 ± 11 % below basal; $n = 4$; $p < 0.05$; Fig. 4a, g), which shows that ACh abolished both the ISO-stimulated and the basal activity of AC. The effect of ACh on ISO-stimulated $I_{Ca,L}$ was mimicked by PKI(15-22) (20 μM) introduced into the myocyte through the patch-pipette by internal perfusion (36 ± 12 % below basal; $n = 4$; $p < 0.05$; Fig. 4b, g). Since $I_{Ca,L}$ in HAMs is often subject to run-down and the effect of PKI(15-22) is irreversible, additional experiments were performed using H-89, a cell-permeable PKA inhibitor. As shown in Fig. 4c, H-89 (10 μM) reversibly inhibited the basal $I_{Ca,L}$ (29 ± 7 % below basal; $n = 4$; $p < 0.01$; Fig. 4g) as well as ISO stimulated $I_{Ca,L}$ (34 ± 7 % below basal; $n = 4$; $p < 0.05$; Fig. 4g), and the effects were similar to those of ACh or PKI(15-22). These experiments indicate that in HAMs, basal $I_{Ca,L}$ is partly carried by PKA-phosphorylated LTCCs, most likely because of a higher basal AC activity. This was confirmed by the observation that either IBMX (100 μM, Fig. 4d) or Cal A (100 nM, Fig. 4e) led to near maximal stimulation of basal $I_{Ca,L}$, respectively to 224 ± 37 % ($n = 5$; $p < 0.005$) and 276 ± 73 % ($n = 4$; $p < 0.05$) of basal level (Fig. 4g). In an attempt to understand why basal AC activity is higher in HAMs, we examined the possibility that β-ARs are constitutively active in these cells by testing the effect of propranolol, an inverse agonist of β-ARs [17]. As shown in Fig. 4f, application of propranolol (1 μM) in HAMs produced an inhibition of basal $I_{Ca,L}$ which was of similar degree as the effect of ACh (18 ± 4.9 % inhibition of basal; $n = 5$; $p < 0.01$; Fig. 4g). In many but not all HAMs, a transient stimulation of $I_{Ca,L}$ was observed upon ACh washout [18, 19], a phenomenon attributed to a rebound stimulation of AC and PKA phosphorylation of LTCCs [19]. Accordingly, adding propranolol during the rebound produced a strong and reversible inhibitory effect of $I_{Ca,L}$ (Fig. 4f). Altogether, these experiments indicate that HAMs possess a stronger basal AC activity than RVMs or FVMs, which is at least partly due to agonist-independent activation of β-ARs.

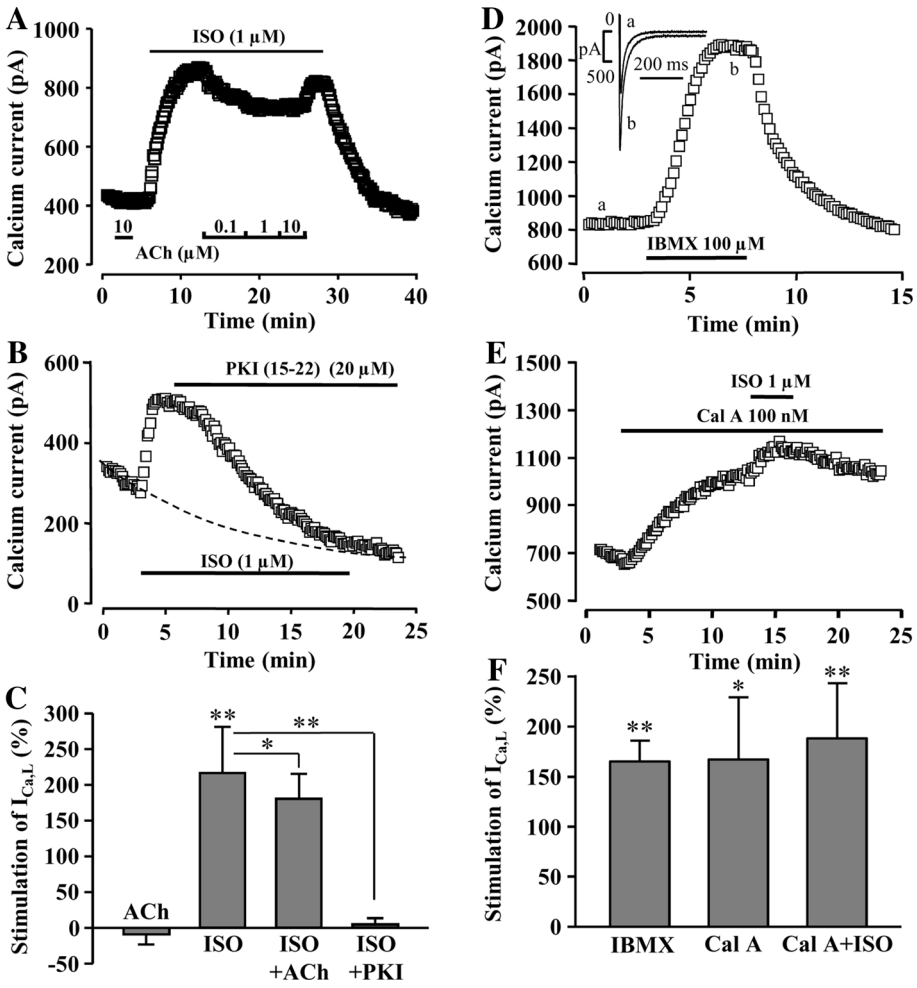

Fig. 3 Control of basal $I_{Ca,L}$ by β-AR-PKA signaling cascade in RVMs. **a** ACh alone had no effect on the basal $I_{Ca,L}$ and dose-dependently but incompletely inhibited ISO-stimulated $I_{Ca,L}$. **b** After stimulation of $I_{Ca,L}$ with ISO (1 μM), the intracellular solution was changed to one containing 20 μM PKI(15-22), and the current returned to basal level. The *dotted line* indicates spontaneous run-down. **c** *Bar graphs* representing the average effect of ACh alone and ISO, alone or in the presence of either ACh or PKI(15-22), on $I_{Ca,L}$. IBMX (100 μM) **(d)** and Cal A (100 nM) **(e)** near maximally stimulate basal $I_{Ca,L}$. When the myocyte was superfused with Cal A and when the current was at its maximal stimulation, ISO (1 μM) induced only a small additional stimulation of $I_{Ca,L}$. The current traces shown in **d** were recorded at the times indicated by the *corresponding letters on the main graphs*. **f** *Bar graphs* representing the average effect of IBMX alone and Cal A, alone or in presence of ISO, on $I_{Ca,L}$. $*p < 0.05$, $**p < 0.01$, compared with basal $I_{Ca,L}$ or between indicated groups

Discussion

The results of the current study can be summarized as follows: (1) AC, PDE, PKA and PPases are active in FVMs, RVMs and HAMs under basal conditions; (2) in FVMs, the basal activity of AC can cause the substantial stimulation of $I_{Ca,L}$ only when PPases are inhibited. The inhibition of PDEs also can cause the potentiation of $I_{Ca,L}$ but only in conditions when PPases are inhibited or AC is prestimulated; (3) in RVMs, the basal activity of AC is high but can induce cAMP-PKA-dependent stimulation of $I_{Ca,L}$ only after PDE or PPase inhibition. ACh only partially inhibits the cAMP-PKA-dependent $I_{Ca,L}$ stimulation; (4) in HAMs, the basal AC activity is sufficient to overcome PDE and PPase activities and maintain $I_{Ca,L}$ significantly elevated above basal ("nonphosphorylated") level. AC activation may result from spontaneous activity of β-ARs in patients with chronic heart failure. The basal PDE and PPase activities are high, and inhibition of any of them causes near maximal stimulation of $I_{Ca,L}$. These results are summarized in Table 1. In addition, there was clear difference in the extent of cAMP depended stimulation of $I_{Ca,L}$ between FVMs, RVMs and HAMs. This may be explained by differences in the modes of excitation–contraction coupling in these species. In mammalian cardiomyocytes, Ca^{2+} ions for the activation of contractile apparatus are mobilized from sarcoplasmic reticulum by Ca^{2+}-induced calcium release. In this way, strong contractile response to β-adrenergic stimulation can be triggered by relatively small increase in $I_{Ca,L}$. However, in frog cardiomyocytes, where the sarcoplasmic reticulum is poorly developed and lacks ryanodine channels [20] the main source of Ca^{2+} is L-type Ca^{2+} channels, and, consequently, for the induction of respective β-AR-dependent contractile response much stronger increase in $I_{Ca,L}$ amplitude is required.

Our study demonstrates that a "basal" $I_{Ca,L}$ may have different meanings in different species and/or cardiac tissues depending on the balance of basal activities of major elements of the cascade: β-ARs, AC, PDE, PKA and PPases. Each member of the cascade has several subtypes or isoforms. Up to date, 3 subtypes of β-ARs have been

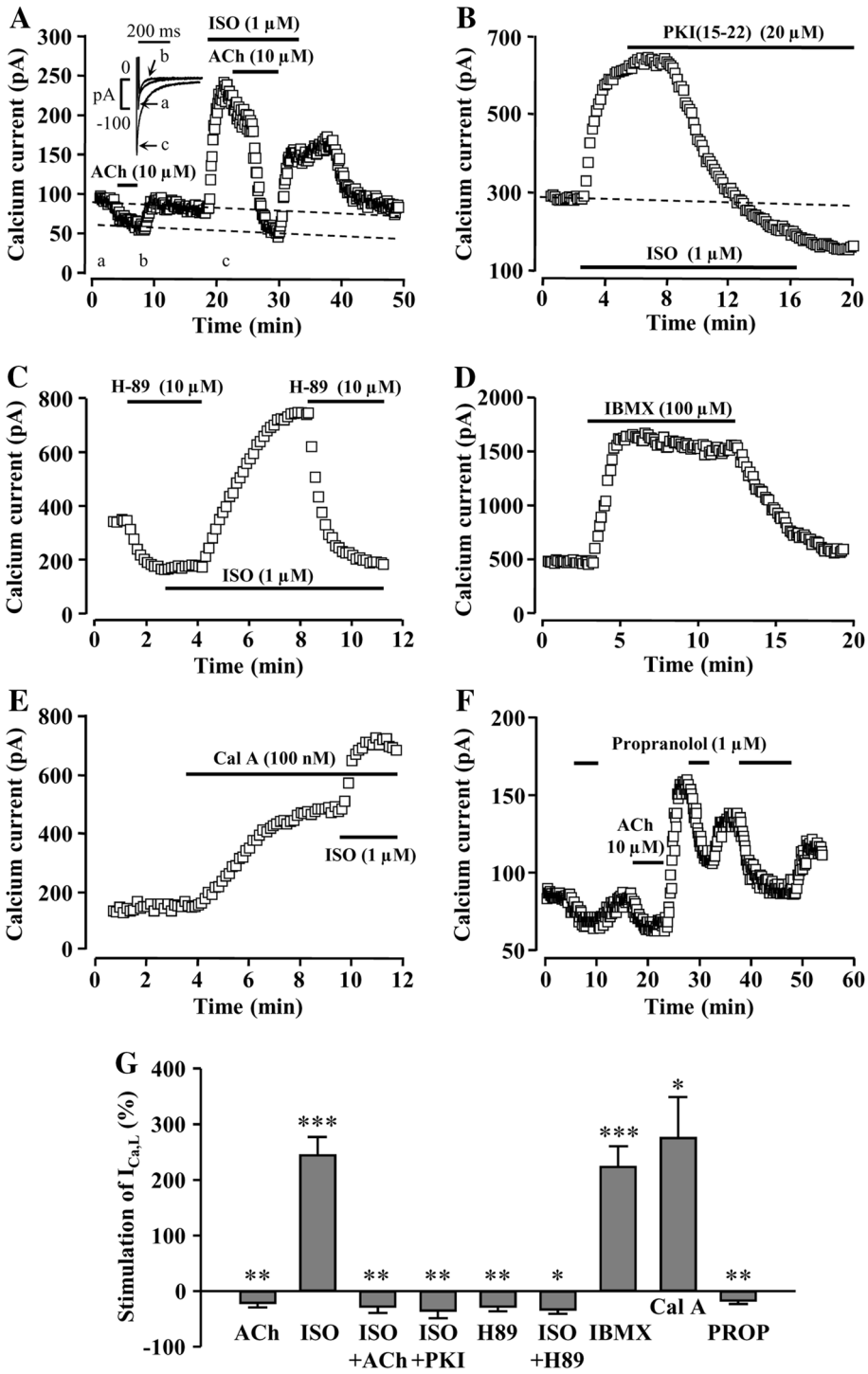

Fig. 4 Control of basal $I_{Ca,L}$ by β-AR-PKA signaling cascade in HAMs. **a** The myocyte was exposed to ACh (10 μM) alone and again after stimulation of $I_{Ca,L}$ with ISO (1 μM). The *dotted line* indicates spontaneous run-down. The current traces shown in the inset were recorded at the times indicated by the *corresponding letters on the main graphs*. **b** After stimulation of $I_{Ca,L}$ with ISO (1 μM), the intracellular solution was changed to one containing 20 μM PKI(15-22), and the current was inhibited below the control level. **c** H-89 (10 μM), a cell permeant inhibitor of PKA, inhibited basal $I_{Ca,L}$ and prevented its stimulation by ISO (1 μM). **d** IBMX (100 μM) near maximally stimulated basal $I_{Ca,L}$. **e** The myocyte was superfused with Cal A (100 nM) and when the current amplitude reached a steady-state, ISO (1 μM) induced additional stimulation of $I_{Ca,L}$. **f** Propranolol (1 μM), an inverse agonist of β-ARs, inhibited basal $I_{Ca,L}$. **g** *Bar graphs* summarizing the effects of all compounds tested in **a–f** on $I_{Ca,L}$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, compared with basal $I_{Ca,L}$

found in the human heart. In addition to a cAMP-dependent effect on $I_{Ca,L}$ common to all three subtypes, β_3-ARs may be involved in the parallel inhibitory NO-dependent pathway [21]. In our earlier study, we have demonstrated that β_3-AR agonists stimulated $I_{Ca,L}$ in HAMs but had no effect on it in FVMs and RVMs [16]. Longer exposure of these receptors, but β_3-AR, to the agonists causes negative feedback due to beta-arrestin, PKA- or GPCR kinase-dependent their desensitization [22–24]. Multiple single nucleotide polymorphisms of all three β-AR subtypes may also modify the signal transduction [25], particularly in certain pathological conditions when density of β_2- [26, 27] and β_3-ARs [28] in the heart may increase over 2–3 times. The same may depend on a variety of GTP-binding protein

Table 1 The basal activities of major elements of β-AR-AC-PKA-dependent signaling cascade

	β-ARs	ACs	PDEs	PKA	PPases	LTCC[a]
FVM	−	±	±	+	+	−
RVM	−	+	+	+	+	−
HAM	+	+	+	+	+	+

[a] indicates the elevation of $I_{Ca,L}$ above basal level

"−" no activity, "±" low activity, "+" high activity

isoforms expressed in different species [29]. Even though these proteins, G_i and G_s, are the major regulators in opposite manner of the activity of all known AC isoforms, many other molecules such as Ca^{2+}, calmodulin, calmodulin kinases II and IV, protein kinase C, phosphatase 2B and others may interfere, as well [30]. So far, 10 isoforms of ACs have been identified (membrane-bound AC(1-9) and soluble AC10) [31]. While AC5 and AC6 are the major AC isoforms in the heart [32], other types, but AC1, in smaller amounts also have been found in the heart of different species and, for instance, AC2, AC4, AC7 were not sensitive to G_i [31] or even might be stimulated by βγ subunits of G_i, provided that G_s was also active [33]. This observation can explain the surprisingly small effect of ACh on ISO-stimulated $I_{Ca,L}$ in RVMs. Alternatively, in the conditions of completely inhibited ACs, ACh might stimulate $I_{Ca,L}$ through the stimulation of cGMP production and inhibition of PDE3 or/and stimulation of M1 receptor-phospholipase C–protein kinase C pathway (for review, see [34]). In physiological conditions, at least 5 isoforms of PDEs may contribute to $I_{Ca,L}$ regulation: Ca^{2+}-dependent PDE1, cGMP-stimulated PDE2, cGMP-inhibited PDE3, cAMP-dependent PDE4 and cGMP PDE5 [35], and possibly PDE8 [36]. In the heart of different species the expression or domination of PDE isoforms may vary [7, 37–39]. Redistribution of PDE2 and PDE3 in the discrete subcellular microdomains has been shown to occur in early cardiac hypertrophy and may cause cGMP-dependent augmentation of contractility [40]. Also, the intrinsic activity of AC, PKA and PDEs is much higher in early than in late developmental stage of the rodent heart [41, 42].

In addition to PPase1 and PPase2A, Ca^{2+}-dependent PPase2B and not well-described PPase2C may play a role in the cascade [43]. The activities of PPases are regulated by some kinases directly or by phosphorylation of phosphatase inhibitors I-1 [44], $I\text{-}1^{PP2A}$, and $I\text{-}2^{PP2A}$ [45]. Compartmentation of cyclic nucleotides (for review, see [5, 46]), different AC isoforms, scaffolding, anchoring, and other cytoskeleton proteins determine the cooperativity of signaling molecules and efficacy of the cascade [47]. Spontaneous cAMP and Ca^{2+} oscillations may occur within microdomains containing exclusively Ca^{2+}-inhibitable AC5 and AC6 [48] which may provide fine tuning of β-AR stimulated $I_{Ca,L}$. Other regulators such as giant sarcolemmal protein AHNAK [49] or as yet unidentified ones interacting with channel subunits may also influence the charge transfer through Ca^{2+} channels. The activity of Ca^{2+} channels may be regulated not only by PKA but also by protein kinases C and G, and tyrosine kinases [50, 51]. The participation of Src family non-receptor tyrosine kinases may be important in determining the state of basal $I_{Ca,L}$ in HAMs of patients with atrial fibrillation [52]. Moreover, our recent paper [50] demonstrates that in HAMs this kinase attenuates PKA-dependent stimulation of $I_{Ca,L}$, a phenomenon we neither observed in FVMs nor in RVMs. Finally, other so far not characterized protein kinases such as protein kinase X, the activity of which in control conditions the first was reported by Hartzell and colleagues [53, 54], may play a role in different species.

In conclusion, our study demonstrates that in the resting physiological conditions the basal $I_{Ca,L}$ and consequently the contractile function of the heart is secured from unnecessary elevation of its activity and energy consumption at the several "checking-points" of cAMP-dependent signaling cascade including adrenergic receptors, G_s and G_i proteins, ACs and PDEs, PKA and PPases. The loading of these "checking-points" may vary in different species and tissues. However, small transient disbalance at the single point not necessary must cause the undesired stimulatory effects due to a double and triple control at the following steps.

Acknowledgments We thank Dr. Rodolphe Fischmeister for valuable discussions, Antanas Navalinskas for skillful technical assistance, and Valeryia Mikalayeva for preparation of the cells. This work was supported by an East–West cooperation grant from INSERM, France.

Conflict of interest The authors declare no competing or financial interests.

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