Establishment of β-Adrenergic Modulation of L-Type Ca\textsuperscript{2+} Current in the Early Stages of Cardiomyocyte Development

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Abstract—β-Adrenergic modulation of the L-type Ca\textsuperscript{2+} current (I\textsubscript{CaL}) was characterized for different developmental stages in murine embryonic stem cell-derived cardiomyocytes using the whole-cell patch-clamp technique at 37°C. Cardiomyocytes first appeared in embryonic stem cell-derived embryoid bodies grown for 7 days (7d). I\textsubscript{CaL} was insensitive to isoproterenol, forskolin, and 8-bromo-cAMP in very early developmental stage (VEDS) cardiomyocytes (from 7+1d to 7+2d) but highly stimulated by these substances in late developmental stage (LDS) cardiomyocytes (from 7+9d to 7+12d), indicating that all signaling cascade components became functionally coupled during development. In early developmental stage (EDS) cells (from 7+3d to 7+5d), the stimulatory response to forskolin and 8-bromo-cAMP was relatively weak. The forskolin effect was strongly augmented by ATP-γ-S. At this stage, basal I\textsubscript{CaL} was stimulated by the nonselective phosphodiesterase (PDE) inhibitor isobutylmethylxanthine, by PDE inhibitors selective for the PDE II, III, and IV isoforms, as well as by the phosphatase inhibitor okadaic acid. Stimulation of I\textsubscript{CaL} by the catalytic subunit of the cAMP-dependent protein kinase A (PKA) was found to be similar (about 3 times) throughout development and in adult mouse ventricular cardiomyocytes, indicating that no structural changes of the Ca\textsuperscript{2+} channel related to phosphorylation occurred during development. I\textsubscript{CaL} was stimulated by isoproterenol in the presence of a PKA inhibitor and GTP-γ-S in LDS but not VEDS cardiomyocytes, suggesting the development of a membrane-delimited stimulatory pathway mediated through the stimulatory GTP binding protein, Gs. We conclude that uncoupling and/or low expression of Gs protein accounted for the I\textsubscript{CaL} insensitivity to β-adrenergic stimulation in VEDS cardiomyocytes. Furthermore, in EDS cells at the 7+4d stage, the reduced β-adrenergic response is due, at least in part, to high intrinsic PDE and phosphatase activities. (Circ Res. 1999;84:136-145.)

Key Words: L-type Ca\textsuperscript{2+} channel ▪ adenylyl cyclase ▪ cAMP-dependent protein kinase A ▪ phosphatase ▪ phosphodiesterase

A n important process in regulating heart rate and excitation-contraction coupling by sympathetic nerves is the stimulation of the high-threshold (L-type) Ca\textsuperscript{2+} current (I\textsubscript{CaL}) by β-adrenergic agonists. In adult mammalian cardiomyocytes, the signal transduction from receptor to the channel includes several steps of interaction of functionally coupled downstream components (Figure 1). Binding of an agonist to the β-adrenoceptor activates a guanine nucleotide-binding (G) protein, Gs, which triggers the activation of adenylyl cyclase (AC) and, in turn, increases the level of cytosolic cAMP.\textsuperscript{1} This is followed by the phosphorylation of the Ca\textsuperscript{2+} channel through the activation of cAMP-dependent protein kinase A (PKA),\textsuperscript{2,3} leading to an increase in channel open probability and channel availability.\textsuperscript{4} However, a direct stimulation of Ca\textsuperscript{2+} channels via Gs protein was also suggested.\textsuperscript{5} Despite recent advances in the understanding of the β-adrenergic modulation of the Ca\textsuperscript{2+} channel during embryonic and postnatal development,\textsuperscript{6} the establishment of this important signaling cascade during cardiomyocyte development is still unresolved. Moreover, studies on isolated cardiomyocytes highlighted the importance of the impaired β-adrenergic modulation in cardiac disorders, such as heart hypertrophy and heart failure.\textsuperscript{8,9} The detailed knowledge about cardiomyocytes of early developmental stages has now become of particular interest, because recent studies show that cardiomyocytes of failing hearts tend to dedifferentiate toward an embryonic phenotype (see References 10 and 11).

On the other hand, dysfunctions in adult cells may stem from activation of a dormant pathway developed earlier during cellular differentiation.

The aim of the present study was to investigate the establishment of the β-adrenergic signaling cascade for early cardiomyocyte development. Because of the known difficulties to obtain cardiomyocytes of the very early developmental stages from the mammalian embryos (eg, before day 12 to 13 of gestation in mouse), we have used for the present study an in vitro model of cardiomyogenesis based on pluripotent mouse embryonic stem (ES) cells. The cardiomyogenesis

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observed in ES cell–derived embryoid bodies (EBs) is almost identical to mouse embryo during early stages of development. Moreover, late-stage ES cell–derived cardiomyocytes exhibit rod-shaped morphology, sarcomere formation, and cell-cell junctions similar to those observed in cardiac myocytes developing in vivo. The electrophysiological characteristics in late developmental stage ES cell–derived cardiomyocytes are identical to postnatal cardiomyocytes. The normal course of development of cardiomyocytes from ES cells has been recently corroborated by the finding that implanted ES cell–derived cardiomyocytes form stable functional grafts in the hearts of adult mice.

The ES cell differentiation model provides the unique possibility to examine the role of particular components involved in signal transduction at different time points during cardiomyogenesis. Early-stage ES cell–derived cardiomyocytes express $I_{Ca}$, as well as the delayed rectifying $K^+$ current. These currents were regulated by both β-adrenergic and muscarinic signaling pathways, suggesting the normal course of development for signaling components. In preliminary experiments, we found a strong developmental increase in the chronotropic response of EBs to β-adrenergic stimulation. Therefore, in the present study, the modulation of $I_{Ca}$ was used as a functional assay to test different components of the β-adrenergic signaling cascade during cardiomyocyte development (Figure 1).

### Materials and Methods

#### Cell Cultures, Differentiation Procedure, and Preparation of Single Cardiomyocytes

The ES cell line D3 was used throughout the present study. These cells were cultivated and differentiated into spontaneously beating cardiomyocytes as previously described. Briefly, ES cells were kept in an undifferentiated, pluripotent state using mitomycin C–inactivated feeder layer of primary cultures of mouse embryonic fibroblasts. They were cultivated on gelatin (0.1%)–coated plastic Petri dishes in DMEM supplemented with 15% FCS (selected batches), l-glutamine (2 mmol/L), and nonessential amino acids. The main steps of differentiation included the following: (1) the cultivation of a definite number of cells (400) in “hanging drops” as EBs for 2 days, (2) cultivation as suspension in bacteriological dishes for 5 days, (3) plating of 7 days’ (7d) EBs on 24–well-microwell plates. About 10% of these 7d EBs already contained contracting cardiomyocytes. In EB outgrowths, cardiomyocytes appeared in the form of spontaneously contracting cell clusters. The clusters increased in size during differentiation. Single cardiomyocytes were isolated from the clusters by enzymatic dissociation with collagenase using a modified procedure of Isenberg and Klöckner described previously. Cardiomyocytes were isolated at 3 distinct developmental stages: (1) the very early developmental stage (VEDS), when first spontaneously contracting clusters of cardiomyocytes appeared (from 7+1d to 7+2d), (2) the early developmental stage (EDS) (from 7+3d to 7+5d), and (3) the late developmental stage (LDS) (from 7+9d to 7+12d). Ventricular cardiomyocytes were isolated from adult mice by collagenase treatment as previously reported.

#### Stable Recording of $I_{Ca}$ by Whole-Cell Patch-Clamp Technique

The whole-cell configuration of the patch-clamp technique was used throughout the present study. This configuration allowed the cell to be dialyzed with various compounds, testing the development of different components of the β-adrenergic signaling cascade. Stimulation effects of $I_{Ca}$ were measured when $I_{Ca}$ rundown was minimized. Fast rundown of $Ca^{2+}$ channel current has been previously reported in embryonic mouse cardiomyocytes when Ba$^{2+}$ was used as a charge carrier. This was also true for cardiomyocytes differentiated from ES cells (authors’ recent unpublished observation). However, we found that $I_{Ca}$ rundown was smaller when Ca$^{2+}$ was used as a charge carrier. Furthermore, the intracellular ATP concentration was raised to 5 mmol/L to minimize $I_{Ca}$ rundown. Under these conditions, the decrease in $I_{Ca}$ amplitude was small particularly during the first 10 minutes after establishment of the whole-cell configuration using 1.8 mmol/L Ca$^{2+}$ as a charge carrier. As previously reported, the size of cardiomyocytes increases during development. For the developmental stages characterized in the present study, VEDS, EDS, and LDS, the average membrane capacities were $19.5 \pm 1.4$ pF (n=16), $29.2 \pm 1.7$ n=39), and $39.2 \pm 2.4$ pF (n=58), respectively. Because cell constituents may be washed out more rapidly in smaller cells and lead to possible artifacts, we ensured that this was insignificant for our investigations on $I_{Ca}$ stimulation. Indeed, the isoproterenol effect on $I_{Ca}$ measured at 5 and 20 minutes after establishment of the whole-cell configuration was identical (difference <10%, n=8; data not shown). Furthermore, although cell dialysis depends even more on pipette...
size, we detected no correlation between $I_{Ca,L}$ stimulation and pipette size (pipette resistances from 0.8 to 4.5 MΩ). Our stable recordings of $I_{Ca,L}$ allowed us to accurately measure and compare effects of $I_{Ca,L}$ stimulation at physiological levels of $[Ca^{2+}]_o$.

**Measurements of $I_{Ca,L}$ Stimulation**

$I_{Ca,L}$ was measured under voltage-clamp conditions by L/M EPC-7 patch-clamp amplifier (List Electronic) or an Axopatch 200A (Axon Instruments) amplifier. Cells were constantly superfused using a gravitational perfusion system, the perfusion rate being $\approx 2$ mL/min. The chamber volume was 0.5 mL. The temperature of the bath as well as of the perforated solutions was kept constant at 37°C. The pipette solution contained (in mmol/L) CsCl 120, MgCl2 3, MgATP 5, EGTA 10, and HEPES 5 (pH 7.4; CsOH). The extracellular solution was of the following composition (in mmol/L): NaCl 120, KC1 5, CaCl2 1.8, TEA-Cl 20, MgCl2 1, and HEPES 10 (pH 7.4; TEA-OH). For some experiments on $I_{Ca,L}$ stimulation, the concentration of $[Ca^{2+}]_o$ was raised to 3.6 mmol/L CaCl2 to increase the amplitude of $I_{Ca,L}$. The internal and external solutions contained Cs+ and tetraethylammonium, respectively, to effectively block K+ current. The $I_{Ca,L}$ peak was measured repetitively at a test potential of 0 mV for 300 or 20 ms from a holding potential of $-40$ or $-50$ mV. For the recording of the time course of peak $I_{Ca,L}$, depolarizing voltage pulses were applied at a frequency of 0.2 Hz. To evaluate the degree of $I_{Ca,L}$ stimulation, the maximum $I_{Ca,L}$ density was taken before and after drug application. The stimulation of $I_{Ca,L}$ is reported in terms of the percentage of the increase of $I_{Ca,L}$ density. The average stimulation effect at a given developmental stage was calculated by averaging individual cell responses. If not stated otherwise, only those cells with an increase of $I_{Ca,L}$ density of $>10%$ after drug application were considered responding cells and included into the statistics. The membrane capacity was measured by applying a voltage ramp by or by using appropriate software (MFK). Current densities are expressed as the $I_{Ca,L}$ peak value per capacity. The results are presented as mean $\pm$ SEM for $n$ cells. The statistical significance of mean values was determined by Student $t$ test for unpaired data. If not stated otherwise, the 2 pools of data were considered to be significantly different at $P<0.01$.

**Investigation of the Establishment of β-Adrenergic Signal Transduction During Development**

The functional coupling of different components of the β-adrenergic signaling cascade was studied during cardiomyocyte development. For this purpose, $I_{Ca,L}$ peak amplitude was measured using different agents. Key molecules involved in signal transduction and specific molecular tools testing their functions are shown schematically in Figure 1. The functional expression of β-adrenergic receptors was tested by extracellular application of isoproterenol (1 μmol/L). The function of AC was tested by its specific activator, forskolin (1 μmol/L). Maximum stimulation of $I_{Ca,L}$ by cAMP was evaluated by cell dialysis with the cAMP analog 8-bromo-cAMP (8-Br-cAMP; 400 μmol/L). The function of phosphodiesterases (PDEs) was tested by using the nonselective inhibitor isobutylmethylxanthine (IBMX) as well as the selective inhibitors EHNA (Erythro-9-[2-hydroxy-3-nonyl]adenine), milrinone, and rolipram for the various PDE isozymes. The receptor-mediated phosphorylation was investigated by coapplication of forskolin (1 μmol/L) and the thiophosphorylating compound ATPγS (2 mmol/L via patch pipette). The effect of Ca2+ channel phosphorylation was tested by cell dialysis with the cationic subunit of PKA (7 μmol/L). ATPγS (1 mmol/L) was added into the pipette solution in addition to the cationic subunit of PKA to make channel phosphorylation irreversible. To test the direct stimulation of $I_{Ca,L}$ via G, protein, isoproterenol was applied extracellularly when cells were dialyzed with 10 μmol/L of PKA inhibitor (PKI) and 1 mmol/L GTPγS. Okadaic acid (10 μmol/L) was used as a phosphatase type 1 and type 2A inhibitor.

**Source of Substances**

Forskolin was purchased from Serva, the catalytic subunit of PKA from Promega, and PKI 5-24 (lot LK-102) from Calbiochem. Cell cultivation medium, FCS, and all other substances for cell cultures were purchased from Gibco BRL. All the other substances were purchased from Sigma Chemical Co.

**Results**

**Isoproterenol, Forskolin, and 8-Br-cAMP Stimulated $I_{Ca,L}$ in LDS but not VEDS Cardiomyocytes**

The effect of isoproterenol on $I_{Ca,L}$ was different in VEDS and LDS cardiomyocytes. Although $I_{Ca,L}$ was insensitive to isoproterenol in VEDS cardiomyocytes (n = 28), $I_{Ca,L}$ was stimulated by isoproterenol in all (n = 24) LDS cardiomyocytes assayed (see examples in Figure 2), suggesting the establishment of a functional β-adrenergic cascade during development. Peak current amplitudes changed insignificantly (remaining within 10% of initial value) during at least 5 minutes after isoproterenol application in VEDS cells (in 25 of 28 cells) within the entire range of tested voltages from $-30$ to 40 mV (Figure 2A). In those 3 responding cells, the average increase in $I_{Ca,L}$ density was very small (16±5%). In contrast, in LDS cells, $I_{Ca,L}$ stimulation amounted to 70±8% (n = 24, measured at 0 mV). The stimulation of $I_{Ca,L}$ was observed in the whole range of test potentials applied, from $-30$ to 40 mV (Figure 2B).

The different sensitivity of the cells to isoproterenol may be a result of the low expression of β-adrenoceptors, as has been previously reported for cardiomyocytes from the fetal murine heart. If the low expression of β-adrenoceptors was the only reason for the unresponsiveness of VEDS cells to isoproterenol, $I_{Ca,L}$ would still be stimulated by either forskolin, an activator of AC or 8-Br-cAMP. We also noticed significant changes of $I_{Ca,L}$ sensitivity to both forskolin (Figure 3A and 3B) and intracellular application of 8-Br-cAMP (400 μmol/L) during development (Figure 3C and 3D). Whereas in VEDS cells forskolin or 8-Br-cAMP did not influence $I_{Ca,L}$, all LDS cardiomyocytes were highly responsive to both drugs. $I_{Ca,L}$ density increase amounted to 82±12% (n = 16) and 156±32% (n = 12) in LDS cells treated with forskolin and 8-Br-cAMP, respectively (see Table for absolute values of $I_{Ca,L}$ densities). This indicated that the full functional coupling of the β-adrenergic signaling cascade was established only in LDS cardiomyocytes. Conversely, the lack of response to isoproterenol, forskolin, and 8-Br-cAMP in VEDS cardiomyocytes (see Figures 2 and 3 and Table) is consistent with a signaling defect at a number of levels in these cells.

**$I_{Ca,L}$ Stimulation in EDS Cardiomyocytes**

To determine the critical period for the development of the functional β-adrenergic signaling cascade, we tested on a “day-by-day” basis the effects of 8-Br-cAMP, isoproterenol, and forskolin on $I_{Ca,L}$ (Figure 4). Most (71%, n = 7) of 7+3d myocytes responded to 8-Br-cAMP exposure with an average increase of $I_{Ca,L}$ density of 18.6±3.2%. Cells of 7+4d stage (79%, n = 14) responded with a significantly larger increase of $I_{Ca,L}$ density (68.2±12.1%). In 7+5d cells, $I_{Ca,L}$ stimulation amounted to 84±21.8% (n = 7, 100% response rate). An important finding was that all cells responded to 8-Br-cAMP after 7+5d, suggesting that significant developmental
changes occur during this short period from day 7+3d to day 7+4d in the EDS cells. However, during this transitory period, the stimulation of $I_{CaL}$ by isoproterenol still remained relatively small, 38.7±5.1% (n=20, 45% response rate, see Figure 4A and Table).

A possible interpretation of these findings in EDS cells was a reduced expression of functional $\beta$-adrenoceptors and/or a defective coupling to AC. We tested therefore different components of the signal transduction cascade in EDS cells to identify the mechanism(s) responsible for the observed changes.
### Development of L-Type Ca\(^{2+}\) Current Regulation

#### Effects of Drugs Testing β-Adrenergic Enzymatic Cascade on \(I_{\text{CaL}}\) Density

<table>
<thead>
<tr>
<th>Drug</th>
<th>VEDS</th>
<th>EDS</th>
<th>LDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7+1d/7+2d</td>
<td>7+3d</td>
<td>7+4d</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>14.4±2.8</td>
<td>13.5±2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.5±3.0</td>
<td>...</td>
<td>18.2±3.6</td>
</tr>
<tr>
<td></td>
<td>n=3 (28)</td>
<td>n=9 (20)</td>
<td>n=24 (24)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>10.9±1.1</td>
<td>14.0±2.2</td>
<td>15.6±2.2</td>
</tr>
<tr>
<td></td>
<td>12.5±1.5</td>
<td>17.8±2.2</td>
<td>21.3±2.6</td>
</tr>
<tr>
<td></td>
<td>n=2 (16)</td>
<td>n=6 (9)</td>
<td>n=8 (8)</td>
</tr>
<tr>
<td>Forskolin+ATP-γ-S</td>
<td>13.8±2.4</td>
<td>9.8±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>21.1±2.9</td>
<td>20.3±1.7</td>
</tr>
<tr>
<td></td>
<td>n=19 (19)</td>
<td>n=14 (14)</td>
<td></td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>9.3</td>
<td>24.8±5</td>
<td>17.2±2.4</td>
</tr>
<tr>
<td></td>
<td>10.6</td>
<td>29±5.1</td>
<td>26.8±2.5</td>
</tr>
<tr>
<td></td>
<td>n=1 (7)</td>
<td>n=5 (7)</td>
<td>n=11 (14)</td>
</tr>
<tr>
<td>PKA catalytic subunit</td>
<td>9.6±0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.6±4.3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>n=6 (6)</td>
<td>n=10 (10)</td>
<td></td>
</tr>
</tbody>
</table>

Shown are average \(I_{\text{CaL}}\) densities before and after drug application. Only those cells that showed increase in \(I_{\text{CaL}}\) density of >10% were considered as responding cells and were used for averaging. Number (n) of responding cells is shown along with total number of cells assayed (in parentheses).

#### Functional Expression of PDEs in EDS Cells

Because 8-Br-cAMP stimulated \(I_{\text{CaL}}\) significantly stronger (about 2 times) than forskolin in 7+4d cells, an involvement of PDEs in \(I_{\text{CaL}}\) degradation was suspected. Therefore, effects of nonselective and selective PDE inhibitors on \(I_{\text{CaL}}\) density were examined (Figure 7). IBMX, a nonselective PDE inhibitor, had a strong stimulatory effect on \(I_{\text{CaL}}\) density (61±7.5%) in 7+3d and 7+4d cardiomyocytes in all cells tested (n=21). The PDE II inhibitor EHNA increased \(I_{\text{CaL}}\) density by 30.2±7.4% in 89% of cells (n=9). The PDE III blocker milrinone stimulated \(I_{\text{CaL}}\) density by 44.2±5.3% in 64% of the tested cells (n=17). Furthermore, the cAMP-dependent PDE IV inhibitor rolipram increased \(I_{\text{CaL}}\) density by 41±9.3% in 69% of cells tested (n=12).

#### Effect of ATP-γ-S and Okadaic Acid on \(I_{\text{CaL}}\) in EDS and LDS Cells

Because coapplication of forskolin plus ATP-γ-S in EDS cells caused a strongly increased stimulation of \(I_{\text{CaL}}\) density compared with forskolin alone, an involvement of phosphatases was suspected. Therefore, the stimulatory effect of intracellular ATP-γ-S (2 mmol/L) application via the patch pipette was tested in EDS and LDS cells (Figure 6A, 6B, and 6D).

ATP-γ-S resulted in a large increase of the basal \(I_{\text{CaL}}\) density (70.3±13%, n=5) in EDS cells, whereas it augmented \(I_{\text{CaL}}\) slightly in LDS cells (9.7±15.6%, n=4, averaging all cells examined). A high intrinsic phosphatase activity in EDS cells was further confirmed by examining the effect of okadaic acid, an inhibitor of type 1 and type 2A phosphatases on \(I_{\text{CaL}}\) density at this developmental stage. Infusion of okadaic acid (10 μmol/L) via the patch pipette resulted in an increase of \(I_{\text{CaL}}\) density by 22±5% (n=5) (Figure 6C and 6D).

#### \(I_{\text{CaL}}\) Was Stimulated by Cell Dialysis With the Catalytic Subunit of PKA in Both VEDS and LDS Cells

The fact that \(I_{\text{CaL}}\) in VEDS cells was not stimulated by forskolin or 8-Br-cAMP indicated that uncoupling of signal transduction at this very early developmental stage could also occur downstream to the β-adrenoceptor or AC, possibly as a result of unresponsiveness of the Ca\(^{2+}\) channel molecule itself. However, \(I_{\text{CaL}}\) was strongly stimulated in all VEDS cells tested (n=6) on intracellular application of the catalytic...
Figure 4. Summary of developmental changes for isoproterenol (A), forskolin (B), and 8-Br-cAMP (C) effects on \( I_{CaL} \). Shown are the percentage of responding cells (with \( I_{CaL} \) increase by >10%), \( \overline{\%} \) and the average \( I_{CaL} \) density increase measured in very early developmental stage (from 7 to 10 days), early developmental stage (from 7 to 3 days), late developmental stage (from 7 to 9 days), and late developmental stage (from 7 to 12 days). The average absolute values of \( I_{CaL} \) densities before and after stimulation are shown in the Table.

Discussion

In the present study, using the stimulation of \( I_{CaL} \) as a functional assay, we have characterized important changes in the functional expression of components of the \( \beta \)-adrenergic signaling cascade occurring during early stages of cardiomyogenesis. Although VEDS cardiomyocytes were insensitive to \( \beta \)-adrenergic stimulation, LDS cardiomyocytes responded to isoproterenol with a large increase of \( I_{CaL} \) density (by \( \approx 70\% \)), similarly as reported for mammalian cardiomyocytes during postnatal development (\( \approx 100\% \)). Our data are in line with reports that embryonic mouse and rat cardiomyocytes are unresponsive to \( \beta \)-adrenergic stimulation.\(^{6,29}\) In addition, the membrane-delimited pathway via direct Ca\(^{2+}\) channel activation by Gs previously reported for adult cardiomyocytes\(^{26,30}\) was absent in VEDS cells.

In the present study, we took advantage of the ES cell–derived cardiomyogenesis to isolate cardiomyocytes at very early developmental stages, when the first contractions occurred.\(^{16}\) Optical recordings of contractions of rat embryos indicated that the paired cardiac primordia are just fused at...
the time of the first spontaneous contractions at the late period of the 3-somite stage. In contrast to the ES cell differentiation system, access to the mammalian heart at such an early developmental stage is complicated. For the VEDS and EDS cardiomyocytes, we developed an experimental protocol of relatively stable recordings of $I_{CaL}$ at physiological Ca$^{2+}$ concentrations and temperature (37°C). The latter is particularly important with respect to the high temperature sensitivity of the regulatory enzyme cascade examined in the present study. This experimental approach allowed the characterization of the mechanisms responsible for changes in the $\beta$-adrenergic modulation of $I_{CaL}$ that occurred during development.

Changes of $I_{CaL}$ Modulation During Development

The lack of $\beta$-adrenergic modulation in VEDS cardiomyocytes suggests that one or more components of the signaling cascade were missing or functionally inactive. To identify these components, we assayed the functional activity of different elements in the $\beta$-adrenergic enzymatic cascade. A key finding was that $I_{CaL}$ was strongly stimulated by cell dialysis with the catalytic subunit of PKA in both VEDS and LDS cardiomyocytes. In contrast to a previous report in which fast current rundown was observed using Ba$^{2+}$ as a charge carrier, we could accurately measure the stimulating effect of the catalytic subunit of PKA on $I_{CaL}$ in VEDS cardiomyocytes. The catalytic subunit of PKA produced a maximal $I_{CaL}$ stimulation. Importantly, $I_{CaL}$ stimulation by the catalytic subunit of PKA was found to be similar ($\approx 3$ times) in VEDS, LDS, and ventricular cardiomyocytes isolated from the adult mouse heart. These data provide strong evidence for similar structural properties of the Ca$^{2+}$ channel or a closely related protein during development at least in regard to phosphorylation sites. Because $I_{CaL}$ was strongly stimulated by the catalytic subunit of PKA but relatively insensitive to either forskolin or 8-Br-cAMP, one explanation for the lack...
of β-adrenergic modulation in VEDS cardiomyocytes could be a low expression of the PKA holoenzyme, as suggested by An et al. 6

The EDS cells proved to be particularly interesting, because forskolin and 8-Br-cAMP already had a stimulatory effect on $I_{\text{Ca,L}}$ density, but the majority of cells still were not stimulated by isoproterenol. Furthermore, we detected a strong stimulation of $I_{\text{Ca,L}}$ by the combined application of forskolin plus ATP-$g$-S. This indicated an involvement of phosphatases in the modulation of $I_{\text{Ca,L}}$ at this developmental stage. Our hypothesis was confirmed by the experimental observation that ATP-$g$-S led to a pronounced stimulation of basal $I_{\text{Ca,L}}$ in EDS cells, whereas at the LDS cell stage only a small stimulation was seen, similarly as reported for guinea-pig ventricular cardiomyocytes.32 These results were further corroborated by experiments using the phosphatase (type 1 and type 2A inhibitor) okadaic acid.33 EDS cells dialyzed with okadaic acid displayed an increase of $I_{\text{Ca,L}}$ density, suggesting that there is an intrinsic AC activity as well as the functional expression of PKA leading to the phosphorylation of Ca$^{2+}$ channels at rest besides an intrinsic phosphatase activity. Because of the intrinsic AC activity, we tested whether cAMP degradation was controlled by intrinsic PDE activity. Indeed, application of the nonselective PDE inhibitor IBMX caused a strong increase of $I_{\text{Ca,L}}$ density. The use of selective PDE inhibitors confirmed the functional expression of the cGMP-dependent type II and III and the cAMP-dependent type IV PDE isoforms. Thus, EDS cells are characterized by high intrinsic AC activity, which is counterbalanced by high intrinsic activity of PDEs and phosphatases.

The strong $I_{\text{Ca,L}}$ stimulation by β-adrenergic agonists in the presence of ATP-γ-S has been previously demonstrated in adult cardiomyocytes.34 Interestingly, forskolin plus ATP-γ-S led to a much stronger stimulation compared with forskolin alone in 7+4d cells, whereas in 7+3d cells this effect was significantly less pronounced. This suggests that at 7+3d, not all β-adrenergic signaling components are fully functional, and, therefore, the role of phosphatases is less important. One day later, however, nearly full coupling is established, and a strong functional role of phosphatases in the dephosphoryla-
tion of stimulated I_{C\alphaL}, even more pronounced than in LDS cells, is observed.

We also tested for the establishment of the membrane-delimited pathway for I_{C\alphaL} regulation via direct interaction of G, and the Ca\(^{2+}\) channel. This modulatory pathway was found only in LDS but not VEDS cardiomyocytes. One of the possible reasons for the lack of both the membrane-delimited and the cAMP-mediated functional coupling of \(\beta\)-adrenoceptors may be a low level of the G protein.\(^{35}\) This is in line with the observation that in the mouse embryonic heart, \(\beta\)-adrenoceptors appear before a detectable heart rate response to isoproterenol.\(^{35}\) Also, important developmental changes in the coupling between \(\beta\)-adrenoceptors and G proteins have been recently reported for fetal rat heart.\(^{36}\)

### Possible Physiological Significance

Functional abnormalities of the cardiac sympathetic nervous system during development were suggested to be involved in the genesis of cardiac arrhythmias, particularly in sudden infant death syndrome.\(^{37}\) Depressed function of \(\beta\)-adrenoceptors,\(^{38}\) deficient production of cAMP,\(^{39}\) and altered expression of G proteins\(^{40}\) are characteristic for cardiomyocytes of failing hearts. Furthermore, an altered coupling of G proteins was suggested to account for decreased stimulation of I_{C\alphaL} by \(\beta\)-adrenergic agonists in hypertrophied\(^{41}\) and diabetic\(^{41}\) hearts. The \(\beta\)-adrenergic receptor system in heart failure is markedly desensitized due to, at least in part, altered expression of \(\beta\)-adrenergic receptor kinase acting in concert with an inhibitor protein, \(\beta\)-arrestin.\(^{42}\) In addition, cardiomyocytes from failing hearts have an altered program of gene expression. Many studies demonstrate that cardiac hypertrophy as well as heart failure is associated with reexpression of an ensemble of genes characteristic of the embryonic heart.\(^{10,11}\) These findings clearly show that detailed knowledge about the establishment of the \(\beta\)-adrenergic signaling cascade during development is important for a better understanding of abnormalities in signaling detected in heart disease. Because cardiac disease states are characterized by hyporesponsiveness to \(\beta\)-adrenergic agonists, it is tempting to speculate that high PDE as well as phosphatase activities may contribute, similar to our findings in EDS cells, to this functional disorder.

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### References


Establishment of β-Adrenergic Modulation of L-Type Ca\(^{2+}\) Current in the Early Stages of Cardiomyocyte Development

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