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Review

# Molecular aspects of adrenergic modulation of cardiac L-type $Ca^{2+}$ channels

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**Abstract**

L-type $Ca^{2+}$ channels are predominantly regulated by β-adrenergic stimulation, enhancing L-type $Ca^{2+}$ current by increasing the mean channel open time and/or the opening probability of functional $Ca^{2+}$ channels. Stimulation of β-adrenergic receptors (ARs) results in an increased cyclic adenosine monophosphate (cAMP) production by adenylate cyclase (AC) and consequently activation of protein kinase (PK) A and phosphorylation of L-type $Ca^{2+}$ channels by this enzyme. $\beta_1$-Adrenergic receptors couple exclusively to the G protein Gs, producing a widespread increase in cAMP levels in the cell, whereas $\beta_2$-adrenergic receptors couple to both Gs and Gi, producing a more localized activation of L-type $Ca^{2+}$ channels. Other signaling intermediates (protein kinase C, protein kinase G or protein tyrosine kinase (PTK)) either have negative effects on L-type $Ca^{2+}$ current, or they interact with the stimulatory effect of the protein kinase A pathway.
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## 1. Introduction

When Orkand and Niedergerke [1] described an inward $Ca^{2+}$ current in *Science* in 1964, this current was not yet known as the L-type $Ca^{2+}$ current ($I_{Ca-L}$). The earlier work on the fast inward $Na^+$ current during the years after the Second World War in squid axon by Weidmann and Coraboeuf and many others had attracted more attention. Orkand and Niedergerke [1] observed that the inward $Ca^{2+}$ current peaked 'late' (in fact it was only 20–30 ms) after the upstroke of the action potential. This "second inward current" was interpreted as something that nature had found to help transform the very short action potential of nerve tissue and skeletal muscle into a cardiac action potential with its substantial longer duration. It contributed to insight in one of the unique characteristics of the ventricle. The long duration of its action potentials causes equally long refractoriness, which protects against re-entrant arrhythmias [2], but also prevents tetanic type of contractions, incompatible with the cyclic function of the heart. Furthermore, the role of the cardiac $I_{Ca-L}$ was instrumental to the notion that the heart relies almost completely on an intracellular reallocation type of $Ca^{2+}$ homeostasis, very different from that of skeletal muscle. This might suggest that $I_{Ca-L}$ has a relatively late appearance during evolution. This is not the

*Abbreviations:* AR, adrenergic receptor; AC, adenylate cyclase; AKAP, A kinase anchoring protein; ATP, adenosine triphosphate; $Ca^{2+}$, calcium; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAG, 1,2-diacylglycerol; GC, guanylate cyclase; G protein, guanosine 5′-triphosphate (GTP)-binding protein; GSNO, nitrosoglutathione; $I_{Ca-L}$, L-type $Ca^{2+}$ current; $InsP_3$, inositol 1,4,5-triphosphate; NO, nitric oxide; $PIP_2$, phosphatidylinositol 4,5-biphosphate; PK, protein kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; Ser, serine; Thr, threonine

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case. In invertebrate species the inward $Ca^{2+}$ current and the so-called transient outward current are the first membrane currents which appear during early development [3]. Recent studies in mammalian embryonic tissue [4] have confirmed that this also applies to vertebrates and the recent developments in research on embryonic stem cells have corroborated this notion (see Ref. [5] for references). Thus, $I_{Ca-L}$ is also an 'early current' in several types of cells developing in the cardiovascular direction and derived from embryonic stem cells from mouse [6] and man [7] or from murine carcinoma cells [8].

$I_{Ca-L}$ constitutes the dominant factor in mediating positive inotropy in all types of cardiac tissue [9]. It also contributes to physiological frequency regulation in the sinus node [10]. Thirdly, it is an important parameter for the duration of the plateau phase of the action potential and is thereby a major determinant of action potential duration and refractoriness. These three physiological functions are under control of catecholamines of circulating and neurohumoral origin. In this brief review we focus on known and putative sites of adrenergic-induced phosphorylation of the L-type $Ca^{2+}$ channel.

## 2. Structure

Voltage-gated $Ca^{2+}$ channels are heteromultimeric protein complexes. The three-dimensional structure of the bovine cardiac L-type calcium channel has recently been resolved [11] (reviewed in Ref. [12]). The largest subunit (~190–240 kDa) is the poreforming $\alpha_1$ subunit, which is associated with an intracellularly located β subunit (~55 kDa) and a mostly extracellularly located disulfide-linked $\alpha_2\delta$ subunit (~170 kDa). The transmembrane $\alpha_1$ subunit contains four homologous domains (I–IV), each of which is composed of six membrane-spanning α helices (S1–S6) [13–15]. The S5 and S6 segments and the membrane-associated pore loop (P-loop) between them form the central pore through which ions flow down their electrochemical gradient. The P-loop contains four negatively charged glutamate residues that are required for the $Ca^{2+}$ selectivity of the channel [13–17]. The fourth transmembrane segment (S4) in each homologous domain contains a positively charged residue (arginine or lysine) at every third or fourth position. This segment serves as the voltage sensor for gating. Moving outward and rotating under the influence of the electric field after depolarization of the membrane, the S4 segments initiate a conformational change that opens the central pore. Thus, the S4 segment controls switching between open and closed conformations of the channel and thus determines whether current will flow [14–16]. The S6 segments form the receptor sites for the pore-blocking $Ca^{2+}$ antagonist drugs specific for L-type $Ca^{2+}$ channels. These segments, together with a motif in the cytoplasmic linker between domains I and II and a motif in the cytoplasmic C-terminus, also provide voltage-dependent channel inactivation [17]. Several $\alpha_1$ subunits have been identified and the $\alpha_{1C}$ isoform is the one that is expressed at high levels in cardiac muscle, but also in smooth muscle and in the brain [16].

The $\alpha_{1C}$ subunit interacts with accessory subunits and especially the β subunit is required to form fully functional $Ca^{2+}$ channels and/or to alter certain channel properties. Accessory subunits determine the activation and inactivation kinetics of the channels. The β subunit also controls targeting of the $\alpha_{1C}$ subunit to the membrane [17,18]. The cytoplasmically located β subunit is strongly hydrophilic. A highly conserved 18-amino acid sequence in the cytoplasmic loop connecting domains I and II has been identified as the interaction domain of the $\alpha_1$ subunit for the β subunit [13–15,17].

The $\alpha_2\delta$ complex, which is less tightly associated with the $\alpha_1$ subunit, consists of an extracellularly located $\alpha_2$ subunit linked to a hydrophobic membrane-spanning δ subunit. The $\alpha_2$ subunit is very hydrophilic and has many glycosylation sites. The $\alpha_2$ and δ subunits are encoded by a single gene. The mature forms of these subunits are derived by post-translational proteolytic processing, but they remain associated through a disulfide bond [13,14,17,18]. The extracellular $\alpha_2$ subunit interacts with the S5–S6 linker in domain III of the $\alpha_1$ subunit [17].

## 3. Function

### 3.1. Basic function

From all cardiac ion currents the $I_{Ca-L}$ is the most extensively studied. Excellent and extensive reviews on its basic kinetics and interaction with several types of ligands are available [19,20]. $I_{Ca-L}$ links membrane depolarization to contraction of the heart by the fact that the $Ca^{2+}$ ions that enter the cell during the depolarization (see below) give rise to subsequent far more massive $Ca^{2+}$ release from the sarcoplasmic reticulum into the cytosol. The channels are closed at the resting potential, but activate upon depolarization. L-type $Ca^{2+}$ channels are activated at relatively positive voltages, with a threshold at about −30 mV [19,20]. These features are even present at early embryonic stage ([21] and references therein). L-type $Ca^{2+}$ channels are further distinguished by a large single channel conductance, a slow voltage-dependent inactivation, marked regulation by protein kinase (PK)A-dependent pathways, and a specific high affinity for $Ca^{2+}$ channel blockers. These $Ca^{2+}$ currents have been designed L-type, as they conduct large, relatively long-lasting currents [14,16,19,20]. Interestingly, and unlike in neurons, inactivation also occurs when $Ba^{2+}$ has taken the place of $Ca^{2+}$ as charge carrier [22]. Therefore, inactivation cannot solely be due to a rise in intracellular $Ca^{2+}$.

The density of $I_{Ca-L}$ increases fivefold in the first 7 days after birth in primary cultures of newborn rat ventricular

myocytes bridging the gap in density between freshly isolated newborn cells (1.6 pA/pF) and freshly isolated adult cells (8.1 pA/pF) [23]. Such an increase in density has also been demonstrated in rabbit ventricular cells [24]. Interestingly, this increase is not homogeneous over the sarcolemma: the developing T-tubule system strongly expresses L-type $Ca^{2+}$ channels, leading to a threefold higher density in T-tubules compared to the rest of the sarcolemma at least in the rat [25]. The amount of functional L-type $Ca^{2+}$ channels, and maybe also expression, decreases again with aging [26].

### 3.2. Adrenergic receptors

Adrenergic receptors (ARs) are G protein-coupled receptors, which contain seven hydrophobic membrane-spanning α-helical domains. Highest amino acid conservation is present in the transmembrane regions, which determine the specificity of ligand binding. The cytoplasmic regions, which interact with other cellular proteins to mediate various signaling events, have more variability [27].

In the human heart nine AR subtypes exist, which mediate a variety of cellular functions. They are encoded by distinct genes. The most abundant types are the β-ARs. There are three subtypes: $\beta_1$, $\beta_2$ and $\beta_3$. The $\beta_1$-AR and $\beta_2$-AR couple to $G_s$ proteins to activate adenylate cyclase (AC), which mediates the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). This leads to the activation of PKA, which in turn phosphorylates several substrates, including L-type $Ca^{2+}$ channels. The $\beta_2$-ARs also couple to $G_i$ proteins, which counteract the $G_s$ coupled activation of AC, resulting in a reduction of cAMP levels [27–30]. The physiological impact as well as the mechanism of action of $\beta_3$-ARs is less clear, although a more prominent role in heart failure has been suggested. Because $\beta_3$-ARs have been reported to produce negative inotropy in human ventricle, a future therapeutic modality might be their blockade in the setting of heart failure (see Ref. [31] for references).

Three subtypes of the $\alpha_1$-AR have been identified: $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$. The $\alpha_{1A}$-AR is the most abundant in the human heart and is coupled via a $G_q$ protein to the activation of phospholipase C (PLC), which causes formation of $InsP_3$ and DAG. The latter mediates the activation of PKC, which phosphorylates many substrates, including L-type $Ca^{2+}$ channels. Also, three $\alpha_2$-ARs ($\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$) exist in the human heart.

Interestingly, L-type $Ca^{2+}$ channel mRNA levels are increased by β-adrenergic signaling, while α-adrenergic signaling produces the reciprocal effect [32].

### 3.3. Response to adrenergic stimulation

Phosphorylation of L-type $Ca^{2+}$ channels promotes $Ca^{2+}$ influx and thus enhances myocyte contraction. L-type $Ca^{2+}$ channels are regulated by different kinases, including PKA, PKC, PKG and protein tyrosine kinase (PTK) (see Sections 4.1–4.4). They are also regulated by G protein subunits in vitro. Both the cardiac $\alpha_{1C}$ and $\beta_{2a}$ subunits of L-type $Ca^{2+}$ channels have been demonstrated to be direct targets of phosphorylation. Multiple modes of gating have been observed at the single channel level: mode 0 in which channels do not open or open very rarely in response to depolarization, mode 1 in which the probability of opening is low with brief open times, and mode 2 in which the probability of opening is much higher and the openings are long-lasting and the closings are brief [14,33]. The increase in $Ca^{2+}$ currents observed after the activation of PKA are due to an increase in the open state probability of the channel, resulting from a shift in gating mode [17,34].

There is an enormous literature on the effects of catecholamines on $I_{Ca\text{-}L}$ which can be subdivided between data obtained in multicellular preparations and in isolated cells/single channels. Also, a subdivision can be made between the effects of α-adrenergic and β-adrenergic effects. Within the context of this paper it is impossible to review this literature in detail. We wish to underscore here that serum has been reported to inhibit basal $I_{Ca\text{-}L}$ [35] and discrepancies between older literature (often on multicellular preparations) and more recent data (often on (sub)cellular preparations) is in part caused by this confounding factor.

In summary, data obtained in multicellular preparations [36–38] and in isolated cells [20] point to an increase of $I_{Ca\text{-}L}$ by $\beta_1$-adrenergic stimulation (Section 3.5). The peak inward current increases primarily by a decrease of the closed time of the channels. α-Adrenergic stimulation is not as effective as β-adrenergic stimulation in multicellular preparations [36–39]. In isolated cells the direct effects are also controversial [20,40,41] (see Section 3.4). It should be noted, however, that methodological aspects are involved because perforated patch-clamp recordings have demonstrated a clear-cut increase in $I_{Ca\text{-}L}$ after stimulation of the $\alpha_1$-AR [42].

### 3.4. Response to α-adrenergic stimulation

Activation of $\alpha_1$-ARs in adult rat ventricular cells does not affect $I_{Ca\text{-}L}$, but in neonatal rat ventricular myocytes the $\alpha_1$-adrenergic agonist phenylephrine concentration-dependently increases $I_{Ca\text{-}L}$ [43]. This stimulating effect of phenylephrine is reversed by the nonselective $\alpha_1$-AR antagonist prazosin. Clonidine, an $\alpha_2$-AR agonist, has no effect on $I_{Ca\text{-}L}$. The $\alpha_2$-AR antagonist yohimbine and the β-AR antagonist propanolol do not inhibit the effect of phenylephrine on $I_{Ca\text{-}L}$, whereas an $\alpha_{1A}$-AR antagonist, but not an $\alpha_{1B}$-AR antagonist, abolishes the effect of phenylephrine. In the presence of propranolol, the non-selective adrenergic agent norepinephrine also increases $I_{Ca\text{-}L}$ in neonatal rat [43]. These results suggest that the increase in

$I_{Ca\text{-}L}$ in neonatal rat ventricular cells is mediated via $\alpha_{1A}$-ARs, although an inhibition of $I_{Ca\text{-}L}$ in neonatal rat ventricular myocytes in response to phenylephrine has been reported as well [44].

### *3.5. Response to β-adrenergic stimulation*

The effects of stimulation of β-ARs on $I_{Ca\text{-}L}$ are predominant over those of α-ARs. Although three types of β-ARs exist in the human heart [31], the effects of stimulation of $\beta_1$-ARs and $\beta_2$-ARs are more important in the mammalian heart and concern an increase in contractility, heart rate, and amplitude of the cardiac action potential. The increase in $I_{Ca\text{-}L}$ by β-adrenergic stimulation is not caused by a change in single channel conductance or in the number of functional channels, but by an increase in the mean channel open time and the probability of channel opening. Activation of β-ARs results in a shift of gating mode 0 to gating modes 1 and 2 [14,34]. Thereby β-adrenergic stimulation results in an increased number of channels being open at a time, which can explain the increase in $I_{Ca\text{-}L}$.

The non-selective β-AR agonist isoproterenol increases $I_{Ca\text{-}L}$ by augmenting cAMP levels [45,46]. However, the increase in $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels in response to β-AR stimulation also acts as a negative feedback on the AC activity. L-type $Ca^{2+}$ channels are probably already phosphorylated under basal conditions, because the decrease of $I_{Ca\text{-}L}$ by the PKA inhibitor H-89 can be reversed with either forskolin or the PP inhibitor okadiac acid.

The stimulatory effect of the $\beta_2$-AR agonist zinterol on $I_{Ca\text{-}L}$ in frog ventricular myocytes is maximal and not additive to the stimulatory effects of isoproterenol. The PKA inhibitor PKI reverses this effect of zinterol. Therefore, the increase in $I_{Ca\text{-}L}$ via $\beta_2$-ARs probably results from stimulation of AC and phosphorylation of the $Ca^{2+}$ channels by PKA [47].

The $\beta_1$-AR activates $G_s$ proteins, but dual coupling of $\beta_2$-ARs to $G_s$ and $G_i$ proteins in rat ventricular myocytes has been demonstrated [48]. After treatment with the $G_i$ inhibitor pertussis toxin, the $\beta_2$-AR-stimulated increase of $I_{Ca\text{-}L}$ is enhanced, while the effect of $\beta_1$-AR stimulation on these $Ca^{2+}$ currents is unaffected. This indicates that a coupling occurs between $\beta_2$-ARs and $G_i$ proteins, exerting negative feedback on the cellular responses to $\beta_2$-AR stimulation [30,48,49] (but see also Ref. [50]).

There is evidence that β-AR stimulation is also involved in myocyte apoptosis [51]. β-Adrenergic modulation of $I_{Ca\text{-}L}$ via $G_s$ proteins is gradually established during development. In myocytes at early developmental stage, forskolin has a weak stimulatory effect on $I_{Ca\text{-}L}$, whereas isoproterenol has no effect at all. However, within a couple of days these substances become effective both in developing cardiomyocytes derived from embryonic tissues and in the embryos themselves [52,53]. The reduced β-adrenergic response in very early cells is, at least partially, due to the high intrinsic activity of protein phosphatases and phosphodiesterases [52].

## 4. Molecular regulation and intracellular pathways

### *4.1. Regulation of $I_{Ca\text{-}L}$ by protein kinase A*

Activation of β-ARs results in the activation of $I_{Ca\text{-}L}$ (see Section 3.5) along many pathways (see Sections 4.2–4.4). The pathway via PKA, which will ultimately lead to phosphorylation of residues of the channel itself, causes an increase in $I_{Ca\text{-}L}$. Activation of $G\alpha_s$ subunits by β-AR agonists (i) stimulates AC (ii), the enzyme that mediates the conversion of ATP into cAMP (iii). Binding of cAMP to the regulatory subunits of PKA (iv) results in the liberation of the catalytic subunits (v), which phosphorylate specific serine and threonine residues of the L-type $Ca^{2+}$ channel (vi) [13,34]. The localization of AC is close to the L-type $Ca^{2+}$ channels in the T-tubules [54]. There is evidence that the β-AR colocalizes with caveolin3, a component of caveolae [55] and the same has been demonstrated for AC [56]. This needs not be in conflict, because it is possible that caveolae and T-tubules are associated as well [57].

Two forms (of different size) of the main subunit ($\alpha_{1C}$) of the L-type $Ca^{2+}$ channel have been detected: a full-length form of ~240–250 kDa and a C-terminally truncated form of ~190–210 kDa. The full-length rabbit $\alpha_{1C}$ subunit is phosphorylated both in vitro and in vivo by PKA in response to elevated cAMP concentrations, but the truncated channel subunit is not [34,53–61]. In intact cardiac myocytes, the majority of $\alpha_{1C}$ subunits are full-length. The truncated form of the $\alpha_{1C}$ subunit is generated by post-translational proteolytic processing [53]. The C-terminal fragment of 30–50 kDa contains a proline-rich domain, which mediates membrane association. Deletion of either the proline-rich domain or truncation of the C-terminus results in an increase of $I_{Ca\text{-}L}$, which suggests that a region in the C-terminal domain has an inhibitory effect on the function of L-type $Ca^{2+}$ channels [62–64].

According to previous literature, the full-length rabbit cardiac $\alpha_1$ subunit contains six potential PKA phosphorylation sites: Ser 124 in the N-terminal part, and five others in the C-terminal part at positions 1575, 1627, 1700, 1848, and 1928. Mutation of Ser 1928 to alanine results in complete loss of cAMP-mediated phosphorylation and in reduction of $I_{Ca\text{-}L}$ [34,65]. The C-terminally truncated $\alpha_{1C}$ subunit lacks Ser 1928 and, thereby, is no longer a substrate for PKA, confirming that, despite the presence of six putative sites, Ser 1928 is the only site, which is in fact phosphorylated by PKA in the $\alpha_{1C}$ subunit [34,59–61]. A previous report on the phosphorylation of the $\alpha_{1C}$ subunit by PKA at Ser 1627 and possibly Ser 1700 [18], has not been confirmed.

Besides phosphorylation, dephosphorylation is also a strictly regulated process. The protein phosphatase inhibitors okadaic acid, microcystin and calyculin A inhibit dephosphorylation of the $\alpha_{1C}$ subunit, albeit in different ways [33,66,67]. Protein phosphatase 2A binds to the 557 amino acids of the C-terminal of the $\alpha_{1C}$ subunit and reverses phosphorylation of Ser 1928 of the L-type $Ca^{2+}$ channel by PKA [33].

None of the important sites phosphorylated by PKA in skeletal muscle are conserved in the cardiac isoform of the channel, and in reverse, the cardiac phosphorylation site (Ser 1928) is not conserved in skeletal muscle $\alpha_{1s}$ [14,18,65].

Besides the $\alpha_{1C}$ subunit, also the $\beta_2$ subunit is a second important target of PKA [68]. PKA still increases $I_{Ca\text{-}L}$ generated by channels with a truncated $\alpha_{1C}$ subunit, when they are associated with a wild type $\beta_{2a}$ subunit [61]. Although the rat $\beta_{2a}$ subunit contains two strong consensus sites for PKA-mediated phosphorylation at Thr 164 and Ser 591, the actual sites of PKA-mediated phosphorylation are at other residues, because mutants that lack both of the consensus sites remain good substrates for phosphorylation by PKA [69]. Phosphopeptide mapping and $\beta_{2a}$ truncation demonstrated that the major sites of PKA-mediated phosphorylation occur at three loose consensus sites for PKA: Ser 459, Ser 478 and Ser 479. Mutation of Ser 459 to alanine results in a reduced rate and degree of phosphorylation of the $\beta_{2a}$ subunit by PKA [69], without altering the basic functional properties of the regulatory $\beta_{2a}$ subunit [61]. Mutation of Ser 478 and Ser 479 to alanine, however, completely abolishes the PKA-induced phosphorylation [69] and prevents PKA-induced $I_{Ca\text{-}L}$ [34,61]. Phosphorylation of the $\beta_{2a}$ subunit at Ser 478 and Ser 479 is pivotal for the regulation of the cardiac L-type $Ca^{2+}$ channel in response to PKA. Phosphorylation of the other associated subunit, the $\alpha_2\delta$ complex, which is less tightly associated with the $\alpha_1$ subunit and consists of an extracellular subunit, has not been detected [34].

For the regulation of the L-type $Ca^{2+}$ channel by PKA, localization of the enzyme to the $Ca^{2+}$ channel is required. PKA is often anchored to specific subcellular compartments by PKA anchoring proteins (AKAPs). These proteins contain a targeting domain that directs the AKAP to a specific cellular site, and a kinase anchoring domain that binds the regulatory subunits of PKA [14,61]. Targeting PKA in close proximity to the L-type $Ca^{2+}$ channel by an AKAP may facilitate phosphorylation of the channel. Anchoring of PKA to the membrane through association with AKAP79 indeed facilitates PKA-mediated phosphorylation of Ser 1928 in the rabbit $\alpha_{1C}$ subunit. AKAP15 directly interacts with $\alpha_{1C}$ through a leucine zipper motif present in the C-terminal tail of the subunit [70]. Phosphorylation of the $\beta_{2a}$ subunit however does not require an AKAP [53,60,61]. Thus, for appropriate PKA-dependent phosphorylation and stimulation of L-type $Ca^{2+}$ channels the enzyme has to be anchored to the membrane by an AKAP. Another important giant sarcolemmal protein (AHNAK) with comparable function has been described as well [71,72].

### 4.2. Regulation of $I_{Ca\text{-}L}$ by protein kinase C

Activation of $G_q$ subunits by $\alpha$-ARs (i) stimulates PLC (ii), which hydrolyzes phosphatidylinositol 4,5-biphosphate ($PIP_2$) to inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) (iii). The latter activates PKC (iv), which in turn phosphorylates many substrates, including L-type $Ca^{2+}$ channels (v) [34].

It has been shown recently that the $\alpha_{1C}$ subunit of the L-type $Ca^{2+}$ channel contains two alternative first exons, exon1a and 1b, which display tissue specific expression in human and rat mediated by alternative promoter usage [73–76]. Exon1a is specifically expressed in cardiac tissue, and codes for a 46 amino acid region of the N-terminus in contrast to the 16 amino acid short N-terminal version coded for by exon1b. The activation of PKC results in a decrease or in a transient increase followed by a decrease of cardiac $I_{Ca\text{-}L}$. Deletion of the initial 46 amino acids of the long version N-terminus of the rabbit $\alpha_{1C}$ subunit increases $Ca^{2+}$ currents [77,78] by increasing single channel open probability with an order of magnitude [77]. Similar findings were observed when comparing the long and short N-terminus form of the human $\alpha_{1C}$ channel [73]. Therefore, the first 46 amino acids of the N-terminus of the $\alpha_{1C}$ subunit have a long-term negative effect on channel gating.

Co-expression of the $\beta_{2a}$ subunit increases $I_{Ca\text{-}L}$, but less in the N-terminal deletion mutant channel than in channels with the full-length $\alpha_{1C}$ subunit. The $\beta_{2a}$ subunit also counteracts the inhibitory effect of PKC. It is proposed that there is an interaction between the $\beta_{2a}$ subunit and the N-terminus of the $\alpha_{1C}$ subunit, resulting in an allosteric competition with the N-terminus to exert its inhibiting effect on gating. The first 5 amino acids of the N-terminus have been identified as very important and the first 20 amino acids as crucial for the inhibitory effect on gating of the $\alpha_{1C}$ subunit of the L-type $Ca^{2+}$ channel gating and for the interaction between the $\alpha_{1C}$ subunit and the $\beta_{2a}$ subunit [79]. Interestingly, none of the first 5 amino acids of the $\alpha_{1C}$ subunit are Ser or Thr. Thus, PKC cannot directly phosphorylate this segment. The N-terminus of the rabbit cardiac $\alpha_{1C}$ subunit contains two putative PKC phosphorylation sites at Thr 27 and Thr 31, but there are conflicting data on the question whether phosphorylation of these sites in fact occurs [78,79] and, if they occur, whether they are relevant for function [34,79]. Conflicting findings have also been observed in studies with direct activators of PKC. It has been suggested that distinct isoforms of PKC may have opposing effects on L-type $Ca^{2+}$ channels [13,34].

### 4.3. Regulation of $I_{Ca\text{-}L}$ by protein kinase G

It is difficult to present a clear outline of the effect of the PKG pathway on the regulation of $I_{Ca\text{-}L}$, because it is not clear whether the effect of PKG induces direct phosphorylation of the L-type $Ca^{2+}$ channel or whether the inhibitory effect of PKG on the PKA pathway, resulting in a decreased cAMP formation, is predominant. Moreover, the cyclic guanosine monophosphate (cGMP)/PKG pathway affects the response to adrenergic stimulation despite the fact that the pathway itself is not directly activated by ARs. Thus, the primary activator of the pathway is not an adrenergic agonist but NO (i), which increases the formation of cGMP from GTP mediated by the cytoplasmic GC (ii). cGMP exerts both stimulatory and inhibitory effects on $I_{Ca\text{-}L}$. This second messenger activates PKG (iii), which either directly phosphorylates the L-type $Ca^{2+}$ channel (iv) or activates a protein phosphatase (v) that dephosphorylates the L-type $Ca^{2+}$ channel (vi). It also stimulates phosphodiesterase 2 (vii), which reduces cAMP levels (viii) and thus inhibits stimulation of the L-type $Ca^{2+}$ channel by PKA [13].

According to previous literature, the rabbit $\alpha_{1C}$ subunit contains two potential PKG phosphorylation sites at Ser 533 and Ser 1371. PKG inhibits rabbit $I_{Ca\text{-}L}$ by phosphorylating the $\alpha_{1C}$ subunit of the channel at Ser 533 [15]. Ser 1371 is located in the fourth transmembrane segment of domain IV of the $\alpha_{1C}$ subunit. So it is not possible that this residue is phosphorylated by PKG in vivo, because only intracellularly located residues are potential targets.

In some cell preparations, a PKG-mediated effect can only be observed after prior activation of the L-type $Ca^{2+}$ channel by PKA [15,80]. The cGMP analogue 8-BrcGMP has no effect on basal single channel gating in mice, but diminishes the PKA-induced activation of L-type $Ca^{2+}$ channels. It still has to be investigated whether cGMP exerts this effect via a direct interaction with PKA or by the activation of PKG [80]. PKG can also activate a sarcolemma bound-protein phosphatase, which dephosphorylates L-type $Ca^{2+}$ channels that were previously phosphorylated by PKA [13]. Finally, there is also evidence that cGMP exerts its inhibitory effect via cGMP-stimulated phosphodiesterase activity, which results in the breakdown of cAMP and subsequent reduction of PKA-mediated increase in $I_{Ca\text{-}L}$ [13,15,81].

In frog ventricular myocytes the NO donor sodium nitroprusside inhibits stimulation of L-type $Ca^{2+}$ channel activity by the β-adrenergic agonist isoproterenol or by the AC activator forskolin via activation of cGMP-stimulated phosphodiesterase-2. The effect is reversed by scavenging NO or by the inhibition of phosphodiesterase-2 [82]. Thus in frog myocytes, stimulation of guanylate cyclase (GC) by NO leads to a reduction of cAMP levels near the L-type $Ca^{2+}$ channels due to activation of phosphodiesterase-2 and thus inhibits stimulation of the L-type $Ca^{2+}$ channel by PKA.

Developmental aspects seem to be involved, because PKG seems to increase basal $I_{Ca\text{-}L}$ in newborn rabbit ventricular cells, but not in adult myocytes. Different isoforms of PKG exist and differing ratios of these isoforms in newborn compared to adult rabbit myocytes may be responsible for different roles of cGMP depending of developmental stages [81].

### 4.4. Regulation of $I_{Ca\text{-}L}$ by protein tyrosine phosphorylation

There is evidence for a role of tyrosine phosphorylation in regulating myocardial β-adrenergic responses, because β-adrenergic stimulation of L-type $Ca^{2+}$ channel activity by isoproterenol is antagonized by a number of phosphotyrosine phosphatase inhibitors [83]. However, no clear overall picture has emerged at this moment. Stimulatory as well as inhibitory effects of PTK inhibitors on $I_{Ca\text{-}L}$ have been reported. For example, the

Table 1
Potential and proven phosphorylation sites of the $\alpha_{1C}$ subunit in different species

| Kinase | Human | Mouse | Rat | Rabbit | Guinea pig |
|---|---|---|---|---|---|
| PKA | Ser124 | – | – | Ser124 | – |
| | Ser495 | Ser495 | Ser495 | Ser495 | Ser494 |
| | Ser528 | Ser528 | Ser528 | Ser528 | Ser527 |
| | Ser893 | Ser893 | Ser893 | Ser893 | Ser892 |
| | Ser1575 | Ser1575 | Ser1575 | Ser1575 | Ser1574 |
| | Thr1604 | Thr1604 | Thr1604 | Thr1604 | Thr1603 |
| | Ser1627 | Ser1627 | Ser1627 | *Ser1627* | Ser1626 |
| | Ser1700 | Ser1700 | Ser1700 | *Ser1700* | Ser1699 |
| | Ser1848 | Ser1848 | Ser1848 | Ser1848 | Ser1847 |
| | Ser1851 | Ser1851 | Ser1851 | Ser1851 | Ser1850 |
| | Thr1900 | Thr1899 | Thr1900 | – | Thr1899 |
| | Ser1912 | – | – | Ser1912 | Ser1911 |
| | Ser1922 | Ser1921 | Ser1922 | Ser1922 | Ser1921 |
| | Ser1928 | Ser1927 | Ser1928 | *Ser1928* | Ser1927 |
| | Ser1974 | Ser1973 | Ser1974 | Ser1974 | Ser1973 |
| | Ser2045 | Ser2045 | Ser2046 | Ser2046 | Ser2045 |
| | Ser2154 | Ser2155 | Ser2156 | – | Ser2155 |
| PKC | – | – | – | *Thr27* | – |
| | Thr31 | – | – | *Thr31* | Thr30 |
| | Thr109 | Thr109 | Thr109 | Thr109 | Thr108 |
| | Thr138 | Thr138 | Thr138 | Thr138 | Thr137 |
| | Ser1627 | Ser1627 | Ser1627 | Ser1627 | Ser1626 |
| | Ser1674 | Ser1674 | Ser1674 | Ser1674 | Ser1673 |
| | Thr1754 | Thr1754 | Thr1754 | Thr1754 | Thr1753 |
| | Ser1843 | Ser1843 | Ser1843 | Ser1843 | |
| | Ser1912 | – | – | Ser1912 | Ser1911 |
| | Ser1945 | Ser1944 | Ser1945 | Ser1945 | Ser1944 |
| | Thr2001 | Thr2000 | Thr2001 | Thr2001 | Thr2000 |
| | Ser2010 | Ser2009 | Ser2010 | Ser2010 | – |
| PKG | Ser533 | Ser533 | Ser533 | *Ser533* | Ser532 |
| | Ser1627 | Ser1627 | Ser1627 | Ser1627 | Ser1626 |
| | Ser1928 | Ser1927 | Ser1928 | Ser1928 | Ser1927 |

Genbank accession numbers are given in the legend of Fig. 1. Phosphorylation sites depicted in italic indicate the availability of experimental data. Consensus motifs: PKA, **R**$X_{1-2}$*/T*X; PKC, XS*/**T***X**R**/**K**; PKG, (**R**/**K**)2−3XS*/T*X (Prosite pattern) [89].

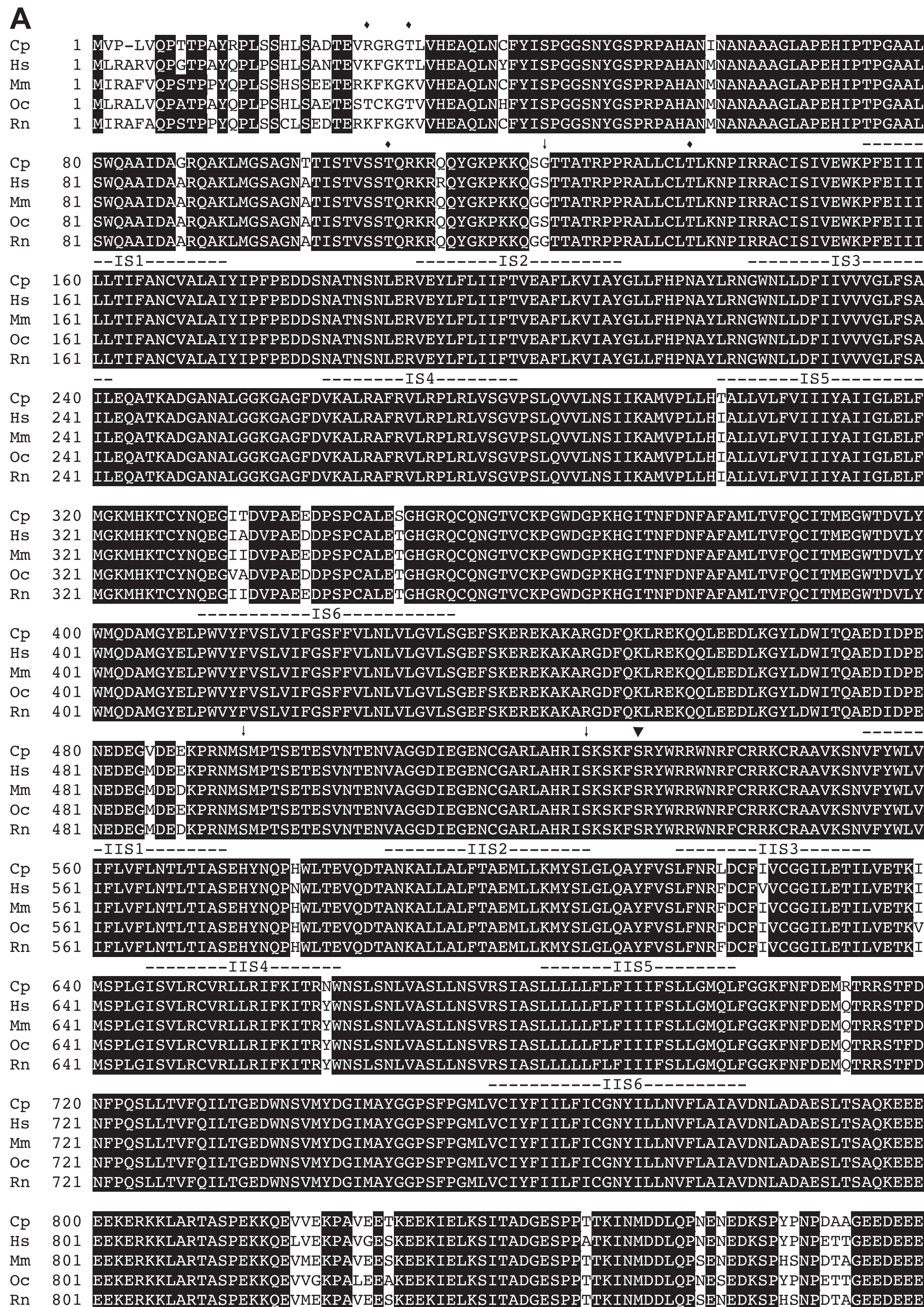



Fig. 1. (A) Comparative alignment of the amino acid sequences of the L-type $Ca^{2+}$ channel $\alpha_{1C}$ subunits. Dashed gaps were introduced to optimize the alignment. The S1–S6 segments of domains I, II, II and IV are indicated. Potential phosphorylation sites (see also Table 1) were labelled as follows. Unique phosphorylation sites: (↓) PKA; (◆) PKC; (▾): PKG. Common phosphorylation sites: (○) PKA and PKC; (●) PKA and PKG; (*) PKA, PKC and PKG. Identical residues for all species are indicated by white lettering over black shading. Abbreviations and accesion numbers: Hs: *Homo sapiens*, AAA17030 and AC005342; Cp: *Cavia porcellus*, AB016287; Mm: *Mus musculus*, NM_009781 and genomic information; Oc: *Oryctolagus cuniculus*, X15539; Rn: *Rattus norvegicus*, AAL47073. (B) Schematic representation of $\alpha_{1C}$. Transmembrane regions are indicated by vertical black thick lines. Phosphorylation sites are indicated as follows: PKA, circles; PKC, triangles; PKG, squares. Open symbols represent potential phosphorylation sites, closed symbols represent phosphorylation sites with experimental evidence.

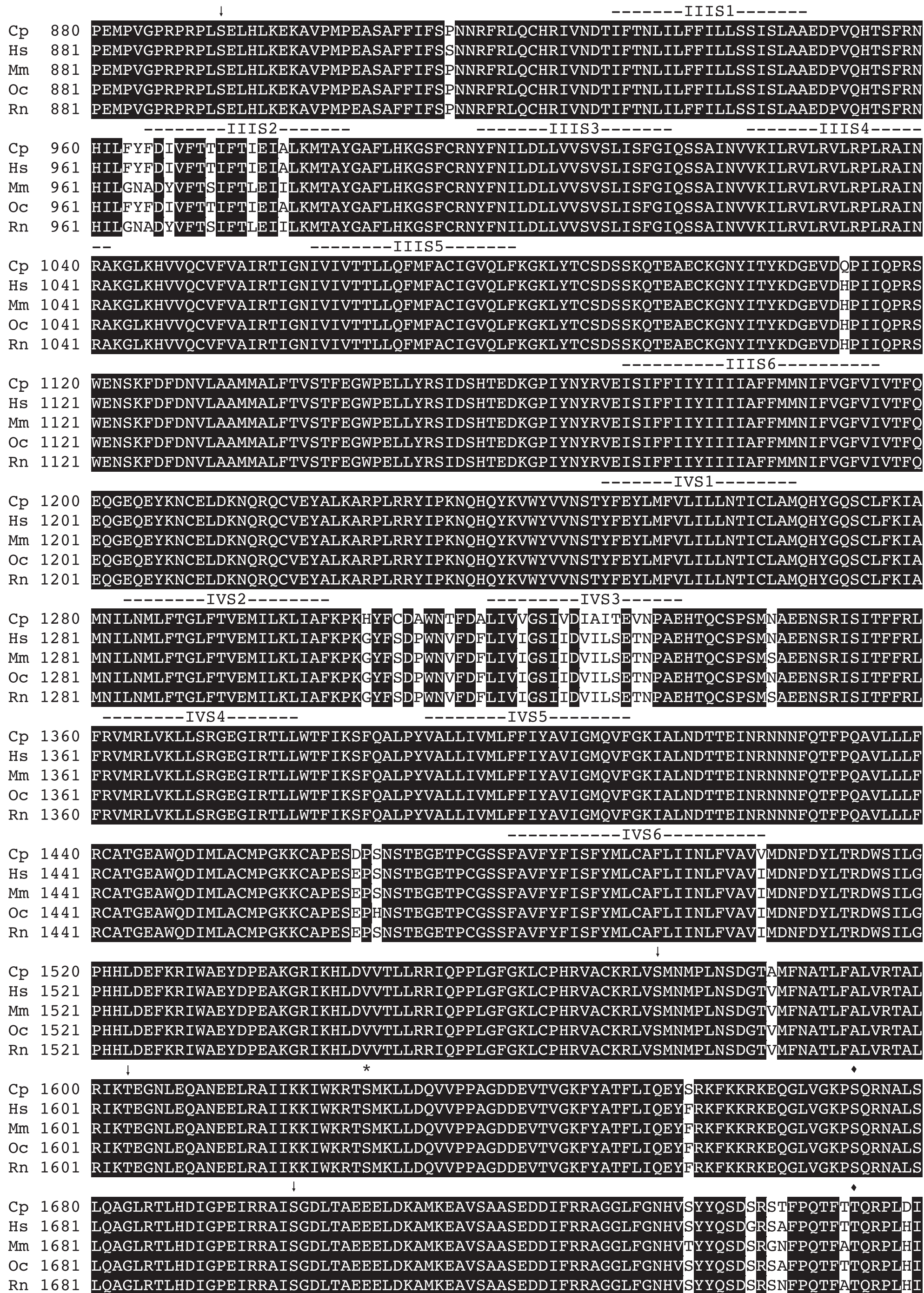
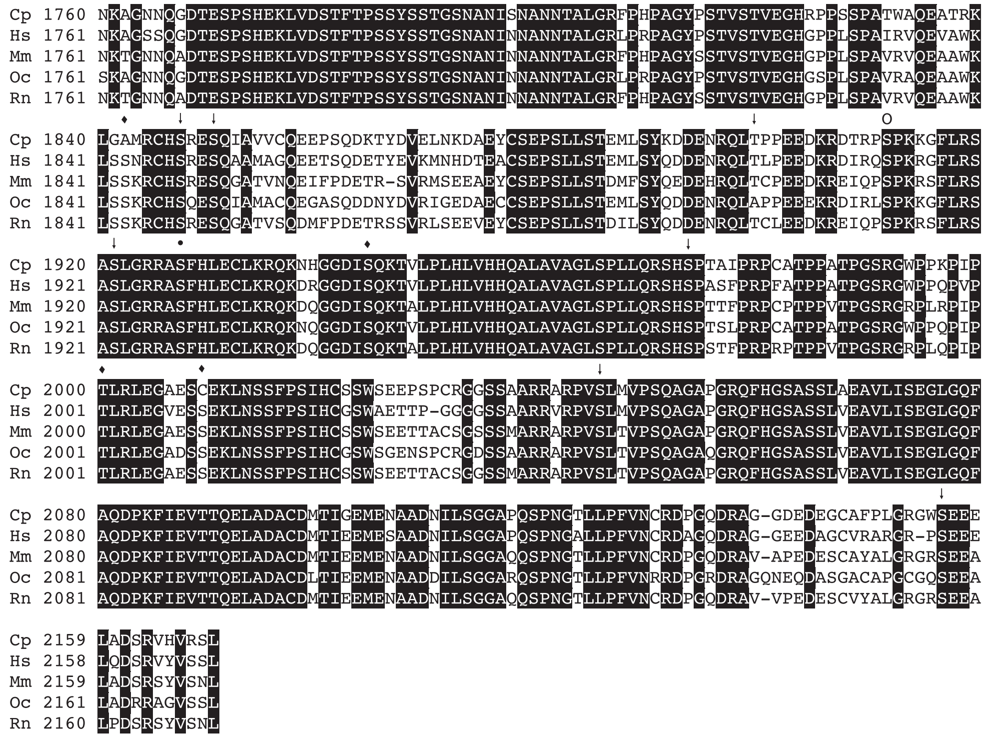


Fig. 1 (*continued*).

PTK inhibitor genistein increases $I_{Ca\text{-}L}$ in human atrial myocytes [84], but reduces $I_{Ca\text{-}L}$ in guinea pig ventricular myocytes [85]. PKC seems to be involved in the mechanism [84]. It has been hypothesized that genistein inhibition of membrane-bound PTK decreases $I_{Ca\text{-}L}$, whereas inhibition of cytosolic PTK increases $I_{Ca\text{-}L}$ [17]. Others report that genistein inhibits $I_{Ca\text{-}L}$ by a tyrosine kinase independent mechanism [86].

## 5. Potential phosphorylation sites of L-type $Ca^{2+}$ channels

There may be more phosphorylation sites in the $\alpha_{1C}$ subunit of L-type $Ca^{2+}$ channels than the ones found in literature. Amino acid sequences from the $\alpha_{1C}$ subunits of different species, containing the exon1a coded region, were retrieved from the GenBank database. The alignment of the



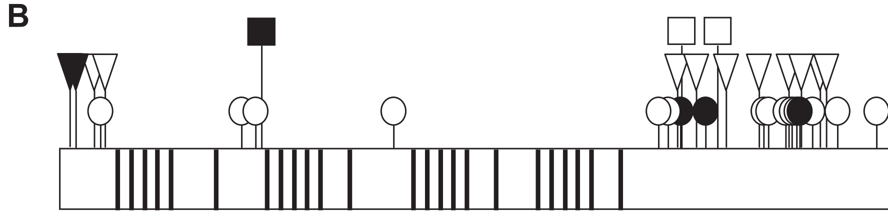




Fig. 1 (*continued*).

amino acid sequences was compared with the alignment made by Mikami et al. [87]. The different domains (extracellular, transmembrane and intracellular) were determined, because only the intracellular domains will be potential targets for PKs. Fig. 1 and Table 1 show the potential phosphorylation sites of PKA, PKC and PKG in man (Hs), guinea pig (Cp), mouse (Mm), rabbit (Oc) and rat (Rn) as determined by using NetPhos (http://www.cbs.dtu.dk/services/NetPhos/). The PK consensus sequences are listed in Table 1. Accession codes of the used sequences are listed in the legend of Fig. 1.

There are (potential) phosphorylation sites, conserved and non-conserved, in literature (see for example Ref. [88]), that were not detected by NetPhos. Thus, there may be more potential phosphorylation sites than the ones presented by us in Table 1. In general, it can be concluded that the potential phosphorylation sites in different species are highly conserved. Remarkably, the established rabbit PKC phosphorylation site Thr 27 [78] is not conserved in other species, while rabbit phosphorylation site PKC Thr 31 [78] is conserved in guinea pig and human, but not in mouse and rat. Instead, positively charged amino-acids are present at these sites, while negatively charged amino-acids would allow PKC mediated inhibition according to McHugh et al. [78].

## 6. Conclusions

L-type $Ca^{2+}$ channels are predominantly regulated by β-adrenergic stimulation, enhancing $I_{Ca\text{-}L}$ by increasing the mean channel open time and/or the opening probability of functional $Ca^{2+}$ channels. Stimulation of β-ARs results primarily in an increased cAMP production by AC and consequently activation of PKA and phosphorylation of L-type $Ca^{2+}$ channels by this enzyme. $\beta_1$-ARs couple exclusively to the G protein $G_s$, producing a widespread increase in cAMP levels in the cell, whereas $\beta_2$-ARs couple to both $G_s$ and $G_i$, producing a more localized activation of L-type $Ca^{2+}$ channels. In neonatal rat ventricular myocytes, $I_{Ca\text{-}L}$ is also regulated by α-adrenergic stimulation, but it still is not clear whether activation of $\alpha_1$-ARs results in activation or in a reduction of $I_{Ca\text{-}L}$. In adult rat ventricular myocytes activation of $\alpha_1$-ARs increases $I_{Ca\text{-}L}$, but only in experiments with the perforated patch-clamp technique. Thus methodological issues at present obscure the physio-

logical significance. The effects of adrenergic stimulation are exerted by phosphorylation of the L-type $Ca^{2+}$ channel subunits by PKA, PKC and PKG.

### 6.1. PKA pathway

Activation of $G\alpha_s$ stimulates AC, which mediates the conversion of ATP into cAMP. This second messenger activates PKA, which increases $I_{Ca\text{-}L}$ via phosphorylation of one or more subunits of the L-type $Ca^{2+}$ channel. In rabbit ventricular myocytes, phosphorylation of Ser 1928 in the $\alpha_{1C}$ subunit is of functional importance for the stimulation of the L-type $Ca^{2+}$ channel in response to PKA. The rat $\beta_{2a}$ subunit is also phosphorylated by PKA at Ser 478 and Ser 479. Phosphorylation of both residues is required for stimulation of the cardiac L-type $Ca^{2+}$ channel. For appropriate phosphorylation of the $\alpha_{1C}$ subunit, PKA has to be anchored to the membrane in close proximity to the L-type $Ca^{2+}$ channel by an AKAP, whereas PKA-dependent phosphorylation of the $\beta_{2a}$ subunit does not require an AKAP.

### 6.2. PKC pathway

Activation of $G\alpha_q$ stimulates PLC, which hydrolyzes $PIP_2$ to $InsP_3$ and DAG. The latter mediates the activation of PKC, which phosphorylates L-type $Ca^{2+}$ channels, but decreases $I_{Ca\text{-}L}$. The first 46 amino acids of the N-terminus of the $\alpha_{1C}$ subunit have a negative effect on channel gating. Phosphorylation of both Thr 27 and Thr 31 of this subunit by PKC inhibits L-type $Ca^{2+}$ channel activity.

### 6.3. PKG pathway

Activation of soluble GC results in the conversion of GTP into cGMP. This second messenger activates PKG, which phosphorylates the rabbit $\alpha_{1C}$ subunit of the L-type $Ca^{2+}$ channel at Ser 533, resulting in an inhibition of L-type $Ca^{2+}$ channel activity. Besides direct phosphorylation of the L-type $Ca^{2+}$ channel, it is also possible that PKG activates a protein phosphatase, which dephosphorylates the channel, or that cGMP activates phosphodiesterase 2, which reduces cAMP levels. Thus stimulation of $I_{Ca\text{-}L}$ by PKA will be inhibited. However, besides an inhibition of $I_{Ca\text{-}L}$, also stimulatory effects of the PKG pathway have been shown.

Using Netphos, the potential phosphorylation sites of the $\alpha_{1C}$ subunit were determined for PKA, PKC, and PKG. The $\alpha_{1C}$ subunits of different species were compared and it can be concluded that the potential phosphorylation sites in different species are highly conserved.

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