*J Physiol* 595.10 (2017) pp 3181–3202



The Journal of Physiology

# Protein kinase A regulates C-terminally truncated $Ca_V1.2$ in *Xenopus* oocytes: roles of N- and C-termini of the $\alpha_{1C}$ subunit

Shimrit Oz[1], Ines Pankonien[2], Anouar Belkacemi[3], Veit Flockerzi[3], Enno Klussmann[2], Hannelore Haase[2] and Nathan Dascal[1,4]

[1] *Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 6997801, Israel*
[2] *Max Delbrück Center for Molecular Medicine (MDC), D-13092, and the German Centre for Cardiovascular Research (DZHK) partner site, Berlin, Germany*
[3] *Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, 66421 Homburg, Germany*
[4] *Sagol School of Neuroscience, Tel Aviv University, Tel Aviv 6997801, Israel*

**Key points**

- $\beta$-Adrenergic stimulation enhances $Ca^{2+}$ entry via L-type $Ca_V1.2$ channels, causing stronger contraction of cardiac muscle cells.
- The signalling pathway involves activation of protein kinase A (PKA), but the molecular details of PKA regulation of $Ca_V1.2$ remain controversial despite extensive research.
- We show that PKA regulation of $Ca_V1.2$ can be reconstituted in *Xenopus* oocytes when the distal C-terminus (dCT) of the main subunit, $\alpha_{1C}$, is truncated.
- The PKA upregulation of $Ca_V1.2$ does not require key factors previously implicated in this mechanism: the clipped dCT, the A kinase-anchoring protein 15 (AKAP15), the phosphorylation sites S1700, T1704 and S1928, or the $\beta$ subunit of $Ca_V1.2$. The gating element within the initial segment of the N-terminus of the cardiac isoform of $\alpha_{1C}$ is essential for the PKA effect.
- We propose that the regulation described here is one of two or several mechanisms that jointly mediate the PKA regulation of $Ca_V1.2$ in the heart.

**Abstract** $\beta$-Adrenergic stimulation enhances $Ca^{2+}$ currents via L-type, voltage-gated $Ca_V1.2$ channels, strengthening cardiac contraction. The signalling via $\beta$-adrenergic receptors ($\beta$-ARs) involves elevation of cyclic AMP (cAMP) levels and activation of protein kinase A (PKA). However, how PKA affects the channel remains controversial. Recent studies in heterologous systems and genetically engineered mice stress the importance of the post-translational proteolytic truncation of the distal C-terminus (dCT) of the main ($\alpha_{1C}$) subunit. Here, we successfully reconstituted the cAMP/PKA regulation of the dCT-truncated $Ca_V1.2$ in *Xenopus* oocytes, which previously failed with the non-truncated $\alpha_{1C}$. cAMP and the purified catalytic subunit of PKA, PKA-CS, injected into intact oocytes, enhanced $Ca_V1.2$ currents by ~40% (rabbit $\alpha_{1C}$) to ~130% (mouse $\alpha_{1C}$). PKA blockers were used to confirm specificity and the need for dissociation of the PKA holoenzyme. The regulation persisted in the absence of the clipped dCT (as a separate protein), the A kinase-anchoring protein AKAP15, and the phosphorylation sites S1700 and T1704, previously proposed as essential for the PKA effect. The $Ca_V\beta_{2b}$ subunit was not involved, as suggested by extensive mutagenesis. Using deletion/chimeric mutagenesis, we have identified the initial segment of the cardiac long-N-terminal isoform of $\alpha_{1C}$ as a previously unrecognized essential element involved in PKA regulation. We propose that the observed regulation, that exclusively involves the $\alpha_{1C}$ subunit, is one of several mechanisms underlying the overall PKA action on $Ca_V1.2$ in the heart. We hypothesize that PKA is acting on $Ca_V1.2$, in part, by affecting a structural 'scaffold' comprising the interacting cytosolic N- and C-termini of $\alpha_{1C}$.



DOI: 10.1113/JP274015

(Resubmitted 6 January 2017; accepted after revision 8 February 2017; first published online 14 February 2017)
**Corresponding author** N. Dascal: Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 6997801, Israel. Email: dascaln@tauex.tau.ac.il

**Abbreviations** AKAP, A-kinase-anchoring protein; AR, adrenergic receptor; cAMP, cyclic AMP; CFTR, cystic fibrosis transmembrane conductance regulator; CS, catalytic subunit; CT, C-terminus; dCT, distal CT; $I_{Ba}$, $Ba^{2+}$ current; LNT, long-N-terminus; NT, N-terminus; PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor protein; RS, regulatory subunit; SNT, short-N-terminus.

## Introduction

The sympathetic system regulates cardiac function, largely through the $\beta$-adrenergic receptors ($\beta$-ARs), causing multiple changes in $Ca^{2+}$ handling and consequently in force, rate and duration of cardiac contraction (Bers, 2008). Increased $\beta$-adrenergic stimulation is harmful in chronic heart failure, and $\beta$-AR-blocking drugs are used to treat a wide range of cardiovascular disorders (Florea & Cohn, 2014). In the classical signalling scheme, adrenaline (epinephrine) or noradrenaline (norepinephrine) binding to $\beta$-AR activates the G protein $G\alpha_s$, stimulating adenylyl cyclase, which increases intracellular levels of cyclic AMP (cAMP). cAMP activates protein kinase A (cAMP-dependent protein kinase, PKA) by dissociating the regulatory subunits (PKA-RS) from the catalytic subunits (PKA-CS). PKA-CS phosphorylates key proteins involved in myocyte contraction, such as the L-type voltage gated $Ca^{2+}$ channel $Ca_V1.2$ in the cardiac sarcolemmal T-tubules, major $Ca^{2+}$-handling proteins of the sarcoplasmic reticulum, certain proteins of the myofilaments, etc. (Wehrens *et al.* 2005; Bers, 2008; Vinogradova & Lakatta, 2009).

The $Ca_V1.2$ channel mediates the fundamental mechanism that initiates the cardiac cell contraction: depolarization-induced $Ca^{2+}$ entry via $Ca_V1.2$ triggers the $Ca^{2+}$-induced $Ca^{2+}$ release from the sarcoplasmic reticulum, which underlies the excitation–contraction coupling (Bers, 2000). $\beta$-Adrenergic stimulation, through the activation of PKA, increases $Ca^{2+}$ entry via $Ca_V1.2$ by enhancing the channel's gating by voltage and increasing its open probability (Osterrieder *et al.* 1982; Reuter *et al.* 1982; Trautwein *et al.* 1982; Cachelin *et al.* 1983; Yue *et al.* 1990). The increased $Ca^{2+}$ influx leads to strengthening of cardiac contraction. This $\beta$-adrenergic action is an important mechanism for regulation of heartbeat by positive inotropic, positive chronotropic and positive lusitropic effects, taking place under a variety of physiological conditions. Nevertheless, despite extensive research, the mechanism linking $\beta$-AR and PKA activation to $Ca_V1.2$ enhancement has not been fully elucidated (reviewed in: Weiss *et al.* 2013; Hofmann *et al.* 2014; Catterall, 2015; Morrow & Marx, 2015).

$Ca_V1.2$ in the heart comprises three subunits: the pore-forming $\alpha_{1C}$, the extracellular $\alpha_2/\delta$, and the cytosolic $Ca_V\beta$ subunit (Catterall, 2000; Dolphin, 2012; Campiglio & Flucher, 2015). Both $\alpha_{1C}$ and $Ca_V\beta$ are phosphorylated by PKA *in vitro* and *in vivo*, and the leading concept is that PKA regulation is mediated by phosphorylation of specific sites in the channel, mainly in the C-terminus (CT) of $\alpha_{1C}$ (reviewed by Catterall, 2015).

The majority of $\alpha_{1C}$ molecules in cardiomyocytes are proteolytically cleaved around amino acid (a.a.) 1800 (Hulme *et al.* 2005, 2006); the exact cleavage site is uncertain (Yang *et al.* 2013). The clipped distal CT (dCT) remains attached non-covalently to the truncated $\alpha_{1C}$, and reduces current density and voltage sensitivity (Wei *et al.* 1994; Klockner *et al.* 1997; Gerhardstein *et al.* 2000; Gao *et al.* 2001; Crump *et al.* 2013). The dCT and its cleavage have been postulated to play an important role in $\beta$-AR modulation of $Ca_V1.2$ in cardiomyocytes (Ganesan *et al.* 2006; Fuller *et al.* 2010; Domes *et al.* 2011; Fu *et al.* 2011, 2013, 2014).

Several mechanisms which assigned crucial roles to various PKA phosphorylation sites in $\alpha_{1C}$ and $Ca_V\beta$ subunits, based on biochemical and heterologous expression data, have been proposed but remained controversial. Inconsistencies between studies prevail, even with the same mammalian cell line such as HEK (reviewed in Weiss *et al.* 2013), presumably due to variability of research methodologies or model systems. The importance of the cardiac cellular environment is not fully understood. Recent studies with animal models carrying targeted mutations and in transfected cardiac cells have challenged most of the proposed mechanisms (Ganesan *et al.* 2006; Lemke *et al.* 2008; Brandmayr *et al.* 2012; Jones *et al.* 2012; Yang *et al.* 2013).

An important proposed mechanism emerged from the reconstitution of $Ca_V1.2$ upregulation by PKA in the tsA-201 mammalian cell line (Fuller *et al.* 2010). Essential requirements for reconstituting the PKA regulation were the truncation of the dCT of $\alpha_{1C}$, the presence of the clipped part of the CT (amino acids (a.a.) 1800–2171) as a separate protein, and the presence of the cardiac A-kinase-anchoring protein AKAP15 (also termed AKAP18$\alpha$; Fraser *et al.* 1998). Mutagenesis suggested that serine 1700 (S1700) and threonine 1704 (T1704) were necessary for the PKA effect in tsA-201 cells, and the proposed model attributed the $Ca_V1.2$ enhancement to a relief of dCT's inhibitory effect upon PKA

phosphorylation of S1700 (Fuller *et al.* 2010). Follow-up studies in a mouse model showed that replacement of serine 1700 or of threonine 1704 by an alanine residue reduced both 'basal' $Ca^{2+}$ currents and sensitivity to $\beta$-AR agonists (Fu *et al.* 2013, 2014). Nevertheless, the two mutations did not fully eliminate the $\beta$-adrenergic regulation (Fu *et al.* 2013). A prominent $\beta$-adrenergic stimulation of $Ca_V1.2$, similar to that seen in wild-type cardiomyocytes, also persisted in a knock-in genetically engineered mouse model with the alanine mutations S1700A and T1704A (Yang *et al.* 2013). The exclusive requirement for AKAP15, or another PKA-anchoring protein, AKAP79, could not be confirmed either, since $\beta$-adrenergic regulation endured in AKAP knock-out mice (Nichols *et al.* 2010; Jones *et al.* 2012). These complex, controversial results may indicate redundancy, i.e. the existence of multiple pathways, of PKA control of $Ca_V1.2$ gating in native cardiomyocytes (Weiss *et al.* 2013). According to this view, some of the pathways suggested by heterologous studies may, in fact, mediate part of the PKA action (such as the phosphorylation of S1700), but their lack in genetically engineered animal models may be compensated by other pathways. Therefore, we posit that, whereas validation in genetically engineered animal models remains essential, further heterologous studies are needed to identify the putative novel or undetected components of the intricate mechanism of PKA regulation of $Ca_V1.2$.

We sought to utilize the experimental approach developed in tsA-201 cells (Fuller *et al.* 2010) to reconstitute and better understand the PKA regulation of $Ca_V1.2$ in *Xenopus* oocytes. Oocytes, like other heterologous systems, lack the specific cellular architecture of cardiac cells which is undoubtedly important for reconstitution of the complete $\beta$-AR cascade. However, they have been successfully used for identification of phosphorylation end-points and additional necessary structural elements in PKA regulation of ion channels such as voltage-gated $Na^+$ channels and the cystic fibrosis transmembrane conductance regulator (CFTR) (e.g. Frohnwieser *et al.* 1997; Wilkinson *et al.* 1997; Csanady *et al.* 2005).

We found that, like in tsA-201, cleavage of dCT was necessary for upregulation of the PKA-dependent $Ca_V1.2$ current. However, PKA-induced enhancement of $Ca_V1.2$ currents did not require the presence of either dCT as a separate protein, AKAP15, $Ca_V\beta$, or the phosphorylation sites S1700 and T1704. The gating element located in the beginning of the N-terminus (NT) of the cardiac isoform of $\alpha_{1C}$ was necessary for cAMP-dependent upregulation of $Ca_V1.2$ currents in *Xenopus* oocytes. These findings suggest that both cytosolic N- and C-termini of $\alpha_{1C}$ participate in the PKA regulation of the channel, pointing to a new direction in the search for pathway(s) of PKA regulation of cardiac $Ca_V1.2$.

## Methods

### Ethical approval

Experiments were approved by Tel Aviv University Institutional Animal Care and Use Committee (permits M-08-081 and M-13-002). Adult female *Xenopus laevis* frogs were purchased from Xenopus-1 (Dexter, MI, USA), transported to Israel and maintained according to the *Guidance on the housing and care of the African clawed frog Xenopus laevis* (Research Animals Department, RSPCA, UK). The frogs were housed in Tel Aviv University Medical School Animal Facility and handled essentially as described (Kahanovitch *et al.* 2014). Female frogs were maintained at 20 ± 2°C on a 10 h light/14 h dark cycle. Frogs were anaesthetized in a 0.17% solution of procainemethanesulphonate (MS222), and portions of ovary were removed through an incision on the abdomen. The incision was sutured and the animal was held in a separate tank until it had fully recovered from the anaesthesia. Afterwards it was returned to a separate tank for post-operational animals. The animals did not show any signs of post-operational distress and were allowed to recover for at least 3 months until the next surgery. Following the final collection of oocytes, anaesthetized frogs were killed by decapitation and double pithing.

### DNA constructs and RNA

The DNA constructs used were: the cardiac long-N-terminus (LNT) isoform of $\alpha_{1C}$ from rabbit (Mikami *et al.* 1989; GenBank: X15539) and the corresponding mouse $\alpha_{1C}$ isoform (NP_001242928; Link *et al.* 2009), $Ca_V\beta_{2b}$ (GenBank: X64297.1; $Ca_V\beta$2N4 according to the comprehensive nomenclature, Hofmann *et al.* 2014), $\alpha_2\delta1$ (GenBank: M21948), AKAP15 (GenBank: AF047716) and enhanced green fluorescent protein (EGFP)-fused Ht31 (Carr *et al.* 1992; Lynch *et al.* 2005). The short-NT (SNT) $\alpha_{1C}$ used here was the $NT_{SL}$ construct (Kanevsky & Dascal, 2006) in which the initial segment of the long-NT (LNT) $\alpha_{1C}$ (encoding the first 46 a.a.) was replaced with that encoded by exon 1 (16 a.a. long). DNAs of rabbit and mouse $\alpha_{1C}\Delta1821$, in which the dCT is truncated, were prepared by the removal of the cDNA segment encoding the dCT from the plasmid and introducing a stop codon after valine 1821, by PCR. NT constructs of rabbit $\alpha_{1C}$ were constructed by sub-cloning into a LNT $\alpha_{1C}\Delta1821$ template (Shistik *et al.* 1998; Kanevsky & Dascal, 2006). Mouse $\alpha_{1C}\Delta5\Delta1821$ DNA (deletion of a.a. 2–5 in the mouse $\alpha_{1C}\Delta1821$ protein) was constructed by PCR procedures and inserted into the pMXT vector. All mouse $\alpha_{1C}$ constructs additionally contained the double mutation T1066Y, Q1070M, which renders the channel dihydropyridine insensitive but does

not affect the regulation by PKA (Yang *et al.* 2013). The following mutations were made in $Ca_V\beta_{2b}$. To construct $Ca_V\beta$-4A, serines 296, 479/80 and 576 were replaced by alanines. To construct $Ca_V\beta_{2b}$-$CT_{trunc}$, a stop codon was introduced after the nucleotide position corresponding to amino acid 471, together with the point mutation S296A (see also Pankonien *et al.* 2012). Mutations were introduced by PCR using the standard site-directed mutagenesis. All constructs were confirmed by DNA sequencing. CFTR (GenBank: M28668) was in a pSP64 vector. All other cDNA constructs used for RNA synthesis were inserted into the pGEM-GSB (Shistik *et al.* 1998) or pGEM-HJ vectors, which are derivatives of pGEM-HE (Liman *et al.* 1992) and contain 5′ and 3′ UTR from *Xenopus* $\beta$-globin. The RNAs were prepared using a standard procedure described previously (Dascal & Lotan, 1992). The amount of injected RNA, per oocyte, for the full-length $\alpha_{1C}$ was 5 ng. For the $\alpha_{1C}\Delta1821$ constructs, the injected RNA amounts were: 0.5–1 ng for LNT, LNTΔ20–46, LNTΔ5 and LNTΔ5Δ20–46, and 0.3–0.5 ng RNA for LNTΔ20, LNTΔ139, SNT, SNT+a.a.2–5 and SNT+a.a.20–46. When $Ca_V\beta$ was not expressed, we injected 7 ng RNA of $\alpha_{1C}\Delta1821$ and $\alpha_2\delta1$.

The following DNA constructs were used for protein purification in *Escherichia coli*: His-tagged C$\alpha$-subunit of PKA (His-PKA-CS, GenBank: NM_008854.5), glutathion-S-transferase (GST)-fused PKA-RII$\beta$ (GST-PKA-RS; GenBank: NM_001030020) and His-tagged human protein kinase inhibitor protein (PKI) (Olsen & Uhler, 1991) (GenBank: S76965.1).

### Protein purification and pull-down assays

Proteins were expressed in *E. coli* (BL21-DE3). For PKA-CS and PKI, respectively, cultures were grown in YT medium at 37°C to an $A_{600}$ of 0.6–0.8 and induced with 0.5 or 1 mM isopropyl $\beta$-D-thiogalactopyranoside for 6.5 h at 24°C (PKA-CS) or for 3 h at 37°C (PKI), before being collected by centrifugation and stored frozen. Pellets were resuspended in buffer I for PKA-CS (in mM: 50 $KH_2PO_4$, 20 Tris-HCl, 100 NaCl, 5 $\beta$-mercaptoethanol, pH 8.0), or buffer II for PKI (in mM: 20 Tris-HCl, 300 NaCl, 0.1% Triton X-100, pH 8.0) with the addition of 15 U $ml^{-1}$ DNase-I, 10 mg lysozyme, 1 mM phenylmethanesulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Cells were lysed using a microfluidizer and clarified by centrifugation. Clarified lysate was loaded onto a Ni-NTA column followed by washing (20 mM) and elution (80 mM) with imidazole. Then the protein was subjected to size-exclusion chromatography on Superdex-75 in buffer III–PKA-CS (in mM: 20 $KH_2PO_4$, 20 KCl, 2 DTT, pH 7.5), or buffer IV–PKI (in mM: 20 Tris-HCl, 300 NaCl, 2 DTT, pH 8). PKI was concentrated in a Vivaspin concentrator with 5000 Da cutoff (Sartorius, Göttingen, Germany). Buffer IV–PKI was used for PKI injection into oocytes (Figs 2 and 3).

For GST-PKA-RS, the pellet was resuspended in PBS supplemented with 0.1% Triton X-100, 15 U $ml^{-1}$ DNase-I, 10 mg of lysozyme, and 1 mM PMSF. The cells were homogenized and lysed with a microfluidizer, and centrifuged for 1 h. The supernatant was loaded onto a glutathione column pre-equilibrated with PBS. Cell extract was loaded on the column and washed with PBS. Protein was eluted with buffer containing (in mM): 50 Tris pH 8.0, 100 NaCl, and 10 glutathione.

Pull-down assays were performed with Ni-NTA resin (Thermo Scientific, Waltham, MA, USA) and washed with buffer containing (in mM): 150 KCl, 50 Tris, 0.6 $MgCl_2$, 1 $CaCl_2$, 0.5% CHAPS, and 10 imidazole, pH 7.4. Elution was done with 100 mM imidazole. Eluates were run on SDS-PAGE gel and stained with Coomassie Blue.

### Electrophysiology

Oocytes were defolliculated by collagenase, injected with RNA and incubated for 2–3 days before recording at 20–22°C in NDE solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, 2.5 pyruvic acid; plus 50 mg $l^{-1}$ gentamycin).

Whole-cell $Ba^{2+}$ currents ($I_{Ba}$) in oocytes were measured using the two-electrode voltage clamp technique (see Fig. 1*A*) with a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA, USA). $I_{Ba}$ was recorded in most cases by 20 ms depolarizing pulses from a resting potential of −80 mV to 20 mV, with a 10 s interval between sweeps, in 40 mM $Ba^{2+}$ solution (in mM: 40 $Ba(OH)_2$, 50 NaOH, 2 KOH, and 5 Hepes, titrated to pH 7.5 with methanesulfonic acid). In some experiments (e.g. Fig. 7), for some $\alpha_{1C}$-derived chimeras, $I_{Ba}$ amplitudes in 40 mM $Ba^{2+}$ solution exceeded 5 $\mu$A. In such cases, to avoid artifacts resulting from oocyte series resistance and poor space clamp, recordings were made in 2 mM $Ba^{2+}$ solution (in mM: 2 $Ba(OH)_2$, 96 NaOH, 2 KOH, and 5 Hepes, titrated to pH 7.5 with methanesulfonic acid). These measurements were used to assess cAMP-induced changes in $I_{Ba}$ amplitude only, and have not been included in amplitude summaries and activation curve fits. In the current–voltage (*I–V*) protocols, currents were elicited by 20 ms pulses from the holding potential −80 mV to voltages from −50 to +60 mV with 10 mV intervals and 10 s between sweeps. Currents measured in the presence of 200 $\mu$M $Cd^{2+}$ were subtracted from total $I_{Ba}$ (Fig. 4*D* and *E*) to yield the net $I_{Ba}$. CFTR currents were measured at −80 mV in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, pH 7.6).

*I–V* curves were fitted to the Boltzmann equation in the form

$$I = G_{max}(V_m - V_{rev})/(1 + \exp(-(V_m - V_a)/K_a)),$$

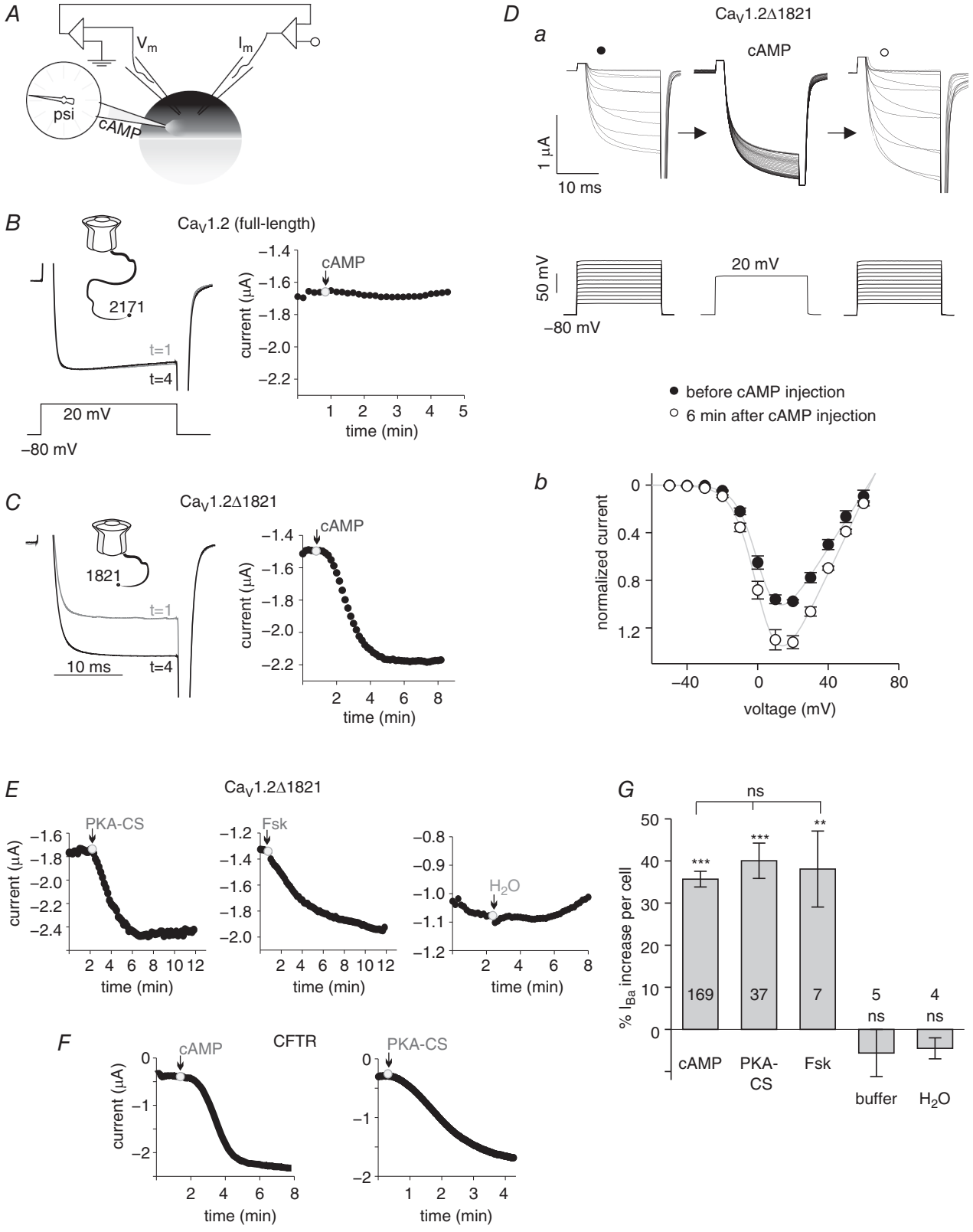


**Figure 1. $Ca_V1.2\Delta1821$ is upregulated by PKA**
*A*, illustration of the setup. The oocyte was impaled with current and voltage electrodes and the injection micropipette. *B* and *C*, cAMP effects on $Ca_V1.2$. Left panels show $I_{Ba}$ of full-length $Ca_V1.2$ (*B*) and $Ca_V1.2\Delta1821$ (*C*)

before cAMP injection (at time 1 min) and 4 min after cAMP injection, in representative cells. Insets above current traces show schematic drawings of the full-length and truncated $\alpha_{1C}$, respectively. Lower inset in *B* shows the voltage step protocol. Right panels summarize the time course of cAMP effect in the same oocytes. cAMP injection is indicated by arrows. Final concentration in the oocyte was 200 $\mu$M. *D*, the *I–V* relations of $Ca_V1.2\Delta1821$ in a representative cell. *a*, the stimulation protocol (lower panel) and the recorded currents (upper panel) before (left) and 6 min after cAMP (right) injection. The middle panel represents the sequence of currents recorded during the 6 min period following cAMP injection; the standard 20 ms voltage pulse was repeated every 10 s. *b*, normalized *I–V* curves (each point shows mean ± SEM), averaged from 5 oocytes. *E*, representative time course charts of $I_{Ba}$, following injection of recombinant PKA-CS, forskolin (Fsk) or water. *F*, representative time course charts of CFTR currents elicited by the injection of cAMP (left, 200 $\mu$M) or recombinant His-PKA-CS (right, 100 nM) into the oocyte. Currents were recorded at −80 mV in ND96 solution. *G*, summary of changes in $I_{Ba}$ in oocytes expressing $Ca_V1.2\Delta1821$ following injection of the indicated compounds. Percentage change was calculated in each cell; number of cells tested is shown within bars. cAMP (200 $\mu$M; summary of 40 experiments); PKA-CS (400 pM to 1 $\mu$M, 11 experiments); forskolin (100 $\mu$M, 1 experiment); Tris buffer (1 experiment); $H_2O$ (1 experiment). Statistical significance (indicated above the bars) of changes in $I_{Ba}$ was calculated for each treatment using paired *t* test. The comparison among all treatments using one-way ANOVA showed no significant difference between groups injected with cAMP, PKA-CS and forskolin. ***$P < 0.001$; **$P = 0.007$; ns, not significant.

where $G_{max}$ is the maximal $Ba^{2+}$ conductance, $V_m$ is the membrane voltage, $V_{rev}$ is the reversal potential of the current, $K_a$ is the slope factor and $V_a$ is half-maximum activation voltage. The parameters obtained for $G_{max}$ and $V_{rev}$ were then used to calculate fractional conductance at each $V_m$ and to construct the conductance–voltage (*G–V*) curve using the equation.

$$G/G_{max} = I/(G_{max}(V_m - V_{rev})).$$

## Injection of cAMP and other substances

In experiments that used pre-injection of the compounds (Figs 2*B* and 3), oocytes were injected with 50 nl of the substance solution 5–30 min prior to recording. Injection during recording was done with sharp capillary glass micropipettes filled with the desired substances (Fig. 1*A*). The compound was pressure-injected into the oocyte as explained in the Results section. The micropipette tip was trimmed so that application of pressure during 1–2 s extruded about 10 nl (1% of oocyte volume). Current stabilized during 1–5 min following insertion of the injection micropipette. Artifacts (sharp shifts in current, e.g. the right panel in Fig. 3*D*) following the insertion of the micropipette or injection of the compound were usually minor. Records with insertion or injection artifacts that exceeded 10% of $I_{Ba}$ amplitude were discarded. The final concentrations of injected substances in the oocytes is indicated in the legends. cAMP (Sigma, A6885) was diluted in $H_2O$ and kept in 1, 20 and 40 mM stock solutions. Injection of 10 nl of cAMP from these stocks gave final concentrations of 10, 200 and 400 $\mu$M in the oocyte, respectively. Purified bovine heart PKA-CS (Sigma, P2645) was diluted in Tris-Mg buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) to 16 units $\mu l^{-1}$. Forskolin (Alomone Labs, Jerusalem, Israel) was diluted in DMSO. Rp-cAMPS (Sigma, A165) was diluted in $H_2O$.

## Giant membrane patches

Giant excised patches of oocyte membrane were prepared as described (Singer-Lahat *et al.* 2000). Oocytes were mechanically devitellinized using tweezers in a hypertonic solution (in mM: 6 NaCl, 150 KCl, 4 $MgCl_2$, 10 Hepes, pH 7.6) and transferred onto a coverslip in EGTA-containing ND96 solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes, 5 EGTA, pH 7.6), with their animal pole facing the coverslip, for 30–45 min. The oocytes were then removed with a jet of solution using a Pasteur pipette, leaving a giant membrane patch attached to the coverslip, with the cytosolic part facing the medium, and the extracellular surface facing the coverslip. The coverslip was washed thoroughly with fresh ND96 solution, and fixated using 4% formaldehyde in EGTA-containing ND96 solution for 30 min. Coverslips were mounted on a glass slide. Giant membrane patches were stained with primary antibody for $\alpha_{1C}$ (1:200; ACC-003, Alomone Labs), followed by incubation with Cy3 fused secondary antibody. The fluorescent labelling was examined by a confocal laser scanning microscope (Zeiss 510 META), using a ×63 oil-immersion objective. Cy3 was excited by a 488 nm laser and the intensities were measured in a 560–569 nm window in the spectral mode. In each experiment, all oocytes from the different groups were studied using a constant set of imaging parameters. Net fluorescence intensity per unit area was obtained by subtracting an averaged background signal measured in the same way on the coverslip outside the oocytes.

## Data presentation and statistics

Imaging data on protein expression (Fig. 4*A*) have been normalized as described previously (Kanevsky & Dascal, 2006). Fluorescence intensity in each giant membrane patch was calculated relative to the average signal in the oocytes of the control group of the same experiment. This

procedure yields average normalized intensity ± SEM in all treatment groups as well as in the control group.

For experiments that involved the injection of substances during current recording, percentage change was relative to the current before injection (which was taken as 100%), in the same cell. The statistical significance of amplitude changes was analysed using a paired *t* test on raw data if normally distributed (using the Kolmogorov–Smirnov test), otherwise a Wilcoxon test was performed. Net CFTR current was defined as $(I_{\text{after injection}} - I_{\text{before injection}})$.

Statistical analysis of data obtained from different test samples (e.g. comparison of currents in oocytes pre-injected with different substances) was performed as follows. Two-group comparisons were performed using Student's *t* test. Multiple group comparison was done with one-way ANOVA if the data were normally distributed. ANOVA on ranks was performed whenever the data did not distribute normally. A Bonferroni *post hoc* test was performed for normally distributed data and Dunn's *post hoc* test otherwise. Unless specified otherwise, the data in the graphs are presented as mean ± SEM and numbers within or near the bars or symbols indicate number of cells tested (*n*). Statistical analysis was performed with SigmaPlot 11 (Systat Software Inc., San Jose, CA, USA).

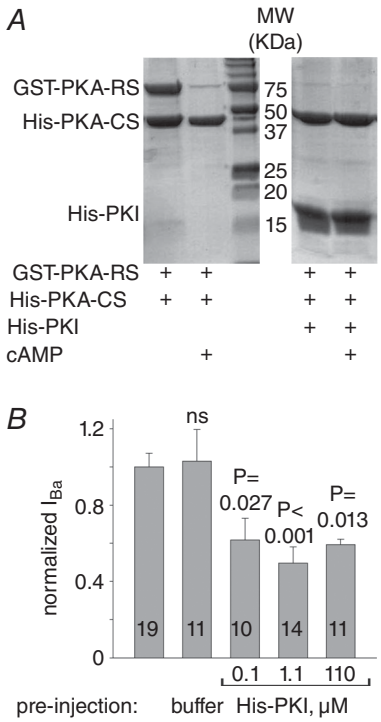


**Figure 2. Purified recombinant PKI inhibits the interaction between PKA-CS and PKA-RS and reduces basal $I_{Ba}$**
*A*, interaction of purified His-PKA-CS (10 µg, 0.8 µM) with GST-PKA-RS (RIIβ, 25 µg, 1.1 µM) was tested in the absence or presence of His-PKI (25 µg, 7.6 µM), with and without 40 µM cAMP. His-tagged proteins were pulled down with nickel beads, eluates were run on SDS-PAGE gel and stained with Coomassie Blue. A representative experiment out of 4 is shown. *B*, PKI reduces basal $I_{Ba}$. Oocytes expressing $Ca_V1.2\Delta1821$ were pre-injected with Tris-NaCl buffer, His-PKI, or not pre-injected. The estimated final concentrations of PKI within the oocyte, assuming a 1 µl cell volume, are shown in µM. Maximal currents in each cell, obtained using an *I–V* protocol of 10 mV depolarization steps from −60 to 50 mV, were averaged and normalized to control group of the same day. Summary from 2 experiments is shown. Statistical significance was determined using one-way ANOVA followed by Bonferroni *t* test.

## Results

### C-terminal truncation of $\alpha_{1C}$ is necessary for PKA upregulation of $Ca_V1.2$ in *Xenopus* oocytes

The majority of previous studies showed that full-length $\alpha_{1C}$ is not significantly upregulated by cAMP or PKA in *Xenopus* oocytes (Singer-Lahat *et al.* 1994; Charnet *et al.* 1995), as well as in mammalian CHO, HEK or tsA-201 cell lines (Perez Reyes *et al.* 1994; Zong *et al.* 1995; Fuller *et al.* 2010). In view of the emerging importance of the C-terminal cleavage of $\alpha_{1C}$ for PKA regulation (Fuller *et al.* 2010; Domes *et al.* 2011; Fu *et al.* 2011), we decided to re-evaluate the effect of PKA on $Ca_V1.2$ in *Xenopus* oocytes using a dCT-truncated $\alpha_{1C}$. We found that this channel is prominently upregulated by PKA (Fig. 1).

We injected cAMP (to activate the endogenous PKA), purified PKA-CS, or other compounds as shown in Fig. 1*A*, and monitored the ensuing changes in $Ca_V1.2$ currents. First, we inserted the voltage and the current electrodes and measured the $Ba^{2+}$ current ($I_{Ba}$) using the two-electrode voltage clamp technique. $I_{Ba}$ was elicited with test pulses to +20 mV every 10 s and continuously monitored during all stages of the experiment. When the current amplitude stabilized, a third micropipette was inserted for pressure injection. The stability of the current amplitude was again verified before the injection of the substances.

We used two distinct $\alpha_{1C}$ constructs: the full-length rabbit cardiac $\alpha_{1C}$ (Mikami *et al.* 1989) (2171 a.a., illustration in Fig. 1*B*), and the C-terminally truncated $\alpha_{1C}$ lacking the dCT after valine 1821, $\alpha_{1C}\Delta1821$ (1821 a.a., Fig. 1*C*). $\alpha_{1C}$ or $\alpha_{1C}\Delta1821$ were expressed, along with the auxiliary subunits $Ca_V\beta_{2b}$ and $\alpha_2\delta1$, by injecting corresponding RNAs at a 1:1:1 ratio. The resulting channels are termed here $Ca_V1.2$ or $Ca_V1.2\Delta1821$, respectively. Consistent with previous reports (Wei *et al.* 1994; Ivanina *et al.* 2000; Gao *et al.* 2001; Hulme *et al.* 2006), $I_{Ba}$ amplitudes in $Ca_V1.2\Delta1821$ were higher compared with the full-length $Ca_V1.2$. In order to maintain similar macroscopic currents, we injected different amounts of the channel RNA: 0.5–1 ng RNA for $Ca_V1.2\Delta1821$ and 5 ng RNA for $Ca_V1.2$.

The modulation of $I_{Ba}$ following the injection of cAMP is illustrated in Fig. 1*B* for $Ca_V1.2$ and in Fig. 1*C* for $Ca_V1.2\Delta1821$. The left panels in Fig. 1*B* and *C* show representative current traces at two time points: $t = 1$ min, just before cAMP injection, and $t = 4$ min, 3 min after cAMP injection. The time course of changes in $I_{Ba}$ in the same cells is shown in the right panels in Fig. 1*B* and *C*. As expected, the full length $Ca_V1.2$ $I_{Ba}$ was not modulated by cAMP. In contrast, $I_{Ba}$ of the C-terminally truncated $Ca_V1.2\Delta1821$ increased following cAMP injection. Figure 1*D* shows the effect of cAMP on current–voltage (*I–V*) relations in a representative oocyte, illustrating the increase in whole-cell $I_{Ba}$ (Fig. 1*Da*) and the ensuing change in the *I–V* curve (Fig. 1*Db*). An

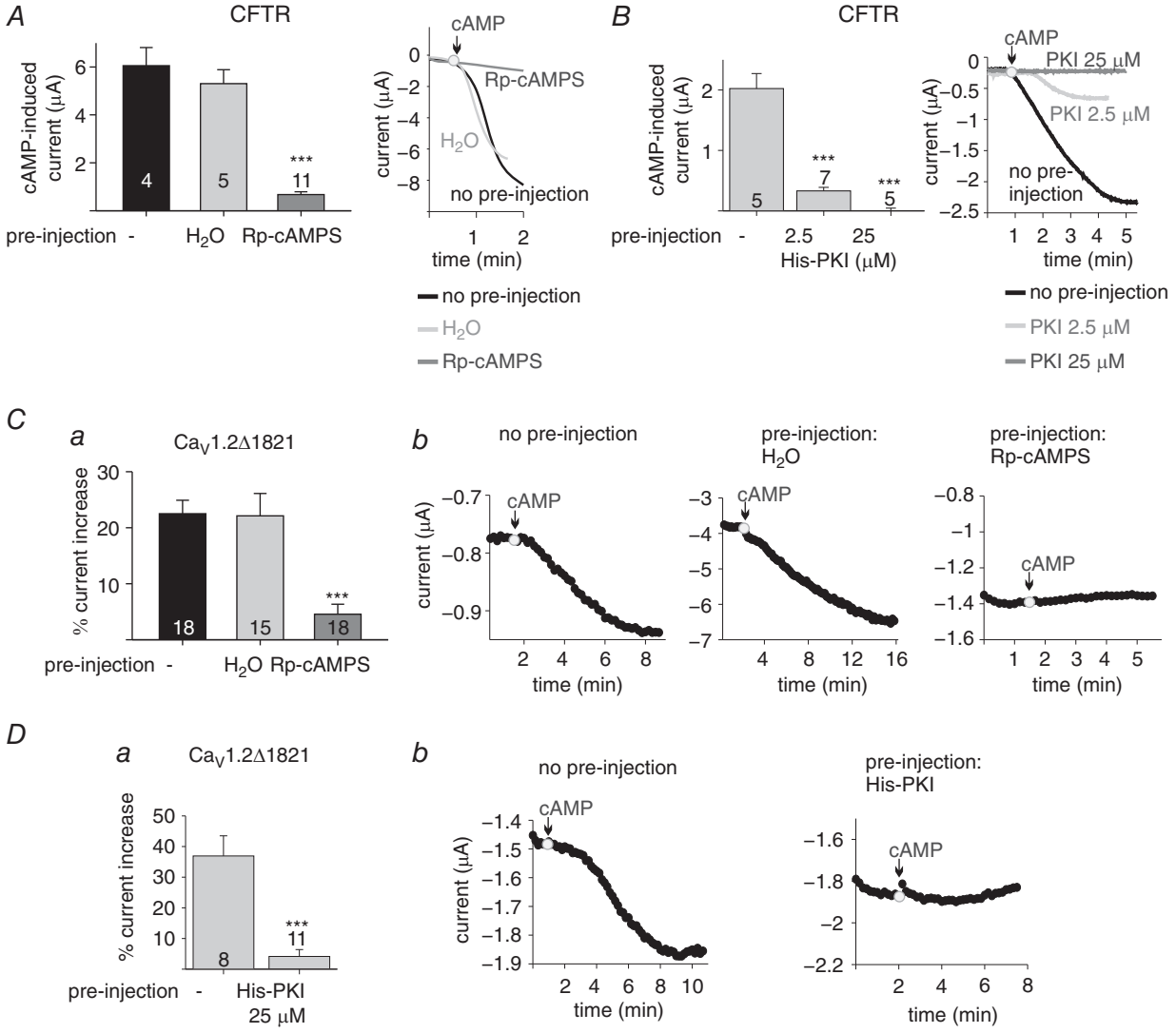


**Figure 3. Rp-cAMPS and PKI inhibit cAMP-dependent upregulation of $I_{Ba}$**
*A* and *B*, effects of PKA blockers on CFTR. Oocytes expressing the CFTR channel were used without any pre-treatment or pre-injected with 50 nl of $H_2O$, Rp-cAMPS (2 mM) (*A*) or PKI (*B*). Oocytes were held at −80 mV and currents were evoked in ND96 solution by injection of cAMP (200 μM). Left, averaged evoked CFTR currents following cAMP injection. Right, representative time course of current changes after cAMP injection. The experiments are from a representative batch of oocytes, out of 3. *C* and *D*, effects of PKA blockers on $Ca_V1.2\Delta1821$. Oocytes expressing $Ca_V1.2\Delta1821$ were used without pretreatment or pre-injected with $H_2O$, Rp-cAMPS (2 mM) (*C*) or PKI (*D*). Following pre-injection, cAMP (200 μM)-dependent changes in $I_{Ba}$ were recorded as in Fig. 1. Averaged % increase in $I_{Ba}$ per oocyte, following cAMP injection (from 3 experiments) is shown in *a*; representative time courses of currents following cAMP injection are shown in *b*. In the set of experiments with Rp-cAMPS, the cAMP-induced increase in $Ca_V1.2\Delta1821$ $I_{Ba}$ was relatively low (22.5% in the control group, less than the average 35.7% summarized from all experiments in Fig. 1*F*), which could reflect low endogenous PKA levels or a high basal phosphorylation of the channel. Statistical significance was determined using one-way ANOVA followed by Bonferroni test, except *Da* where a *t* test was applied. ***$P < 0.001$.

increase in $Ca_V1.2\Delta1821$ $I_{Ba}$ was also recorded following the injection of purified PKA-CS protein or the adenylyl cyclase activator, forskolin (Fig. 1*E*).

We used the cystic fibrosis transmembrane conductance regulator (CFTR) as the positive control for endogenous PKA activation by cAMP, and for PKA-dependent current modulation. CFTR is a chloride channel gated by PKA phosphorylation along with ATP binding (Welsh *et al.* 1992; Gadsby & Nairn, 1999). CFTR's $Cl^-$ current ($I_{Cl}$) is robustly activated by PKA in *Xenopus* oocytes (Bear *et al.* 1991; Uezono *et al.* 1993). As expected, both injected cAMP and PKA-CS activated CFTR and elicited large $I_{Cl}$, usually between 1 and 4 $\mu$A (Fig. 1*F*).

The upregulation in $Ca_V1.2\Delta1821$ currents is summarized in Fig. 1*G* as percentage increase from basal current to current at the time of maximal increase in $I_{Ba}$. Time to maximal increase varied between 5 and 15 min among oocytes. An average 35.7 ± 1.9%

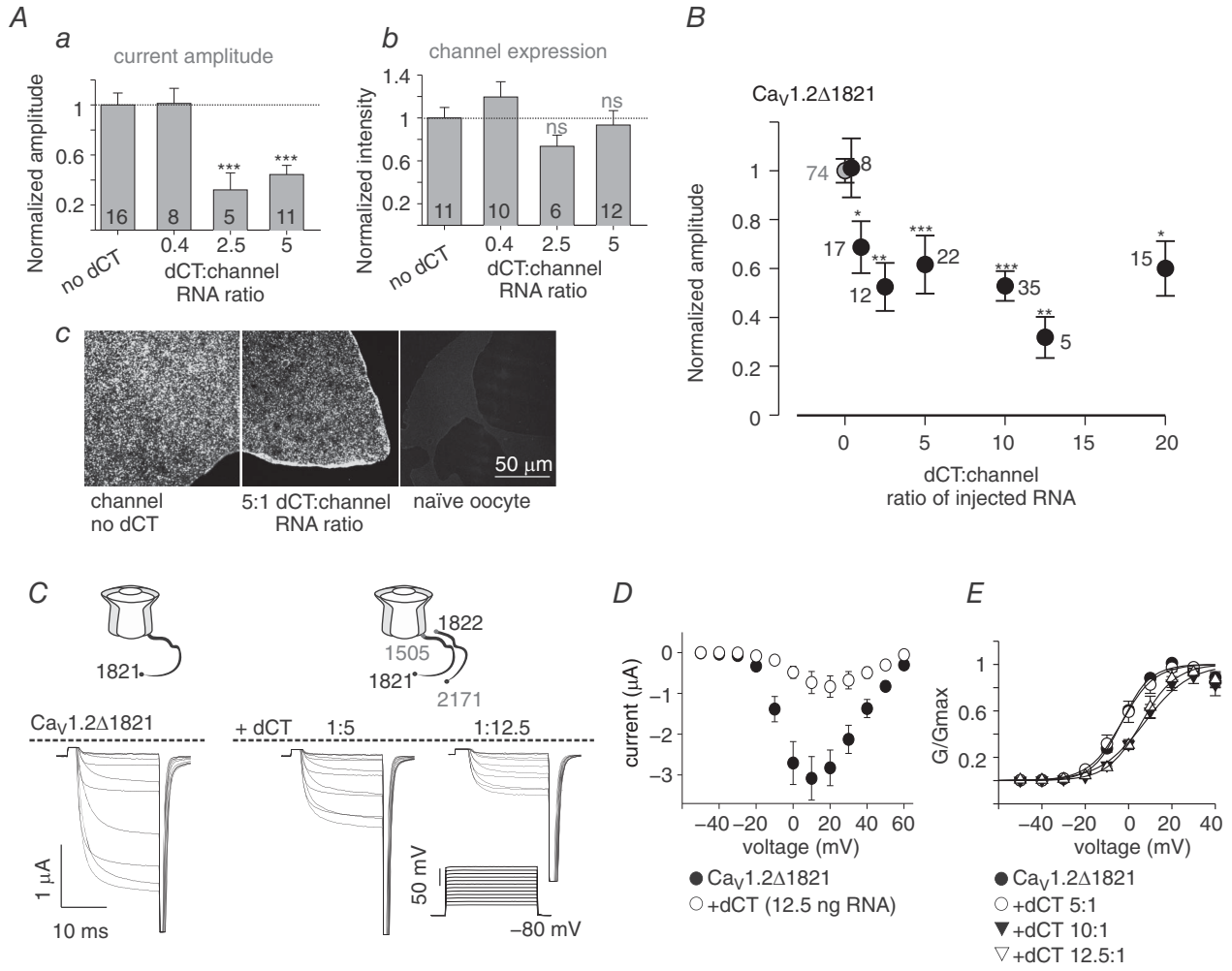


**Figure 4. Overexpression of dCT reduces basal $I_{Ba}$ amplitude and depolarizes the activation curve**
*A*, $I_{Ba}$ amplitudes at +20 mV (*a*) and plasma membrane expression (*b*) were measured in oocytes expressing $Ca_V1.2\Delta1821$ together with increasing levels of dCT. In each experiment both current amplitude and surface labelling intensity were measured and normalized to the group expressing the channel alone. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. ***$P < 0.001$; ns, not significant. $N = 3$ experiments. *c*, examples of confocal images of giant membrane patches, where the channel levels were quantified with $Ca_V1.2$ antibody directed to an intracellular loop. *B*, dose-dependent reduction of $I_{Ba}$ by coexpressed dCT. Oocytes were injected with 1 ng RNA $Ca_V1.2\Delta1821$ together with increasing amounts of dCT RNA. The leftmost grey point represents channels expressed without dCT. In each experiment, $I_{Ba}$ amplitude was normalized to average $I_{Ba}$ in the control group expressing the channel alone. $N = 11$ experiments. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *C* and *D*, current–voltage (*I–V*) relationships in oocytes expressing 1 ng $Ca_V1.2\Delta1821$ and no dCT or 5 or 12.5 ng dCT RNA, from a representative experiment ($n = 5$–7 oocytes). Representative records of net $I_{Ba}$ (*C*) and averaged *I–V* curves (*D*). *E*, averaged conductance–voltage relationships from 3 experiments ($n = 25$ without dCT, and $n = 5$–9 with the different doses of dCT RNA). The Boltzmann equation fit parameters are shown in Table 2.

**Table 1. cAMP at 10 $\mu$M causes maximal activation of CFTR**

| cAMP concentration ($\mu$M) | cAMP-evoked CFTR current (nA) | *n* |
|---|---|---|
| 400 | −1484 ± 132 | 11 |
| 200 | −1776 ± 282 | 8 |
| 10 | −1610 ± 207 | 10 |

cAMP-evoked CFTR current: summary of two experiments; the difference in effect between the three cAMP doses was not significant ($P = 0.611$, one-way ANOVA).

increase in $I_{Ba}$ ($n = 164$; range: 6–100%) was observed following the injection of cAMP to a final concentration of 200 $\mu$M in the oocyte. After injections of purified bovine heart PKA-CS (final concentration in the oocyte 0.16 units $\mu l^{-1}$), $I_{Ba}$ increased by 43 ± 5.5% ($n = 20$). With recombinant His-tagged PKA-CS (see Fig. 2; final concentration 100 nM to 1 $\mu$M), the increase was 36 ± 4.7% ($n = 17$). Figure 1*G* shows pooled data (40 ± 4.2% increase in $I_{Ba}$, $n = 37$; range: 9–114%) for all PKA-CS experiments.

Contrary to cAMP, injection of water or Tris-Mg buffer did not increase $I_{Ba}$ of $Ca_V1.2\Delta1821$ (Fig. 1*E*–*G*). This indicates that the current enhancement of $I_{Ba}$ seen in Fig. 1*C*–*E* is not an injection artifact. The small and statistically non-significant negative change in $I_{Ba}$ reflects the occasional small rundown of $I_{Ba}$ observed following buffer or water injection. This could be happening because of a deterioration of the cell's condition, possibly caused by the insertion of micropipettes.

Our data suggest that the concentrations of cAMP and PKA-CS that we used have produced maximal upregulation of rabbit $Ca_V1.2\Delta1821$ and of CFTR. All three compounds used (cAMP, PKA-CS or forskolin) produced a similar increase in $Ca_V1.2\Delta1821$ current (Fig. 1*F*). Furthermore, in a separate experiment we verified that a 20-fold dilution of cAMP yielded a similar increase in $I_{Ba}$: 33.8 ± 5.5% ($n = 5$) with 10 $\mu$M cAMP *vs.* 33.75 ± 5.1% ($n = 4$) with 200 $\mu$M cAMP in the oocyte. Similarly, 10–400 $\mu$M cAMP induced CFTR currents of comparable sizes (Table 1).

We purified and used recombinant proteins: His-tagged PKA-CS; His-tagged, highly specific heat-stable protein kinase inhibitor protein (PKI) (Walsh *et al.* 1971; Olsen & Uhler, 1991); and GST-tagged PKA-RII$\beta$ regulatory subunit (PKA-RS). To test their functionality, we performed a pull-down assay in which His-tagged PKA-CS, immobilized on nickel agarose beads, bound and pulled down PKA-RS (Fig. 2*A*). In the presence of cAMP, the PKA holoenzyme complex dissociates to a large degree (Beavo *et al.* 1974; Kim *et al.* 2007). In concordance, our pull-down assay showed that in the presence of 40 $\mu$M cAMP, PKA-CS did not pull-down PKA-RS. PKI is a PKA-CS pseudo-substrate that competes with the PKA-RS on binding to the active site cleft in PKA-CS and also blocks the catalytic subunit directly (Scott *et al.* 1985; Knighton *et al.* 1991; Kim *et al.* 2005). When added, His-PKI completely eliminated the interaction between PKA-CS and PKA-RS (Fig. 2*A*). These experiments attest to the quality and functionality of the recombinant proteins used in our experiments.

The basal $I_{Ba}$ may be composed of current from non-phosphorylated and endogenously phosphorylated channels. To measure the contribution of phosphorylated $Ca_V1.2\Delta1821$ to basal $I_{Ba}$, we injected 156 $\mu$M His-PKI, 5–20 min before measuring $I_{Ba}$. This caused a 40% reduction in $I_{Ba}$ compared to oocytes pre-injected with buffer alone (Fig. 2*B*). Thus, about 40% of the amplitude may be due to endogenous PKA activity. To calculate the full span of the enhancing effect of PKA on $Ca_V1.2$ in our experiments, we took into account the PKA-dependent part of the basal current. A 40% increase in current due to cAMP or PKA-CS, on top of only 60% of the basal current, would yield a 1.4/0.6 = 2.3-fold increase of rabbit $\alpha_{1C}\Delta1821$ current due to PKA-CS activity.

To further examine the specificity of the cAMP and PKA-CS effects, we used the PKI protein (a direct inhibitor of PKA-CS) and an indirect PKA inhibitor, the cAMP diastereoisomer Rp-cAMPS, which competes with cAMP on the PKA-RS binding sites and inhibits the dissociation of PKA-CS from PKA-RS (Anand *et al.* 2010). First, we validated the experimental protocol with CFTR as a PKA activation reporter. We pre-injected the inhibitors, and 5–30 min later measured the cAMP-induced CFTR $Cl^-$ currents. Rp-cAMPS was injected to obtain a final concentration of 2 mM, which is comparable to 1–5 mM used by others to inhibit the $\beta$-adrenergic regulation of $Ca_V1.2$ in whole-cell experiments in cardiomyocytes (Hartzell *et al.* 1991; Hirayama & Hartzell, 1997). Figure 3*A* shows that Rp-cAMPS inhibited the cAMP effect on CFTR by 89%, while pre-injection of $H_2O$ did not significantly alter the cAMP effect. Pre-injection of purified His-PKI to 2.5 $\mu$M (final concentration in the oocyte) yielded an 84% inhibition of cAMP-induced CFTR current, and a complete inhibition was achieved at 25 $\mu$M His-PKI (Fig. 3*B*). This concentration range is similar to that employed by others who used PKI or PKI peptides to inhibit $\beta$-adrenergic regulation of $Ca_V1.2$ in cardiomyocytes (Kameyama *et al.* 1986; Parsons *et al.* 1991; Hartzell *et al.* 1995; Skeberdis *et al.* 1997).

Next, we examined the effects of PKA inhibitors on cAMP regulation of $Ca_V1.2\Delta1821$ using the same experimental protocol as for CFTR. Rp-cAMPS inhibited 80% of the cAMP-induced effect. Pre-injection of water did not alter the cAMP effect (Fig. 3*C*). Similarly, the cAMP-induced increase in $I_{Ba}$ of $Ca_V1.2\Delta1821$ was reduced by 89% when oocytes were pre-injected with

His-PKI to a final concentration of 25 $\mu$M (Fig. 3*D*). Taken together, the results so far suggest that cAMP-induced upregulation of both CFTR and $Ca_V1.2\Delta1821$ channel currents in oocytes is a PKA-specific modulation that requires dissociation of PKA-CS from PKA-RS.

## dCT and AKAP15 are not essential for cAMP upregulation of $Ca_V1.2\Delta1821$ in *Xenopus* oocytes

The results of Fig. 1 demonstrate that truncation of the dCT of $\alpha_{1C}$ is essential for the PKA effect on $Ca_V1.2$. However, the presence of the 'clipped' dCT seems non-obligatory. To better understand the role of dCT in cAMP modulation of the truncated $Ca_V1.2\Delta1821$ in *Xenopus* oocytes, we coexpressed $Ca_V1.2\Delta1821$ with the dCT as a separate protein. We first examined whether the coexpression of dCT produces the two characteristic effects on the truncated $Ca_V1.2$: a reduction in maximal inward $I_{Ba}$ amplitude and a depolarizing shift of the activation (conductance–voltage, *G–V*) curve (Hulme *et al.* 2006; Fuller *et al.* 2010; Crump *et al.* 2013).

Coexpression of dCT, at variable ratios of RNA concentrations of dCT and $Ca_V1.2\Delta1821$ ('dCT:channel RNA ratio'), caused a large reduction of 60–70% in $I_{Ba}$ amplitude when using 2.5:1 to 5:1 dCT:channel RNA ratios (Fig. 4*Aa*). The expression of dCT in the same batch of oocytes did not alter the channel density in the plasma membrane, as demonstrated using immunocytochemistry in giant excised plasma membrane patches (Fig. 4*Ab* and *c*). Thus, the reduction in $I_{Ba}$ following coexpression of dCT resulted from a modification of the gating properties, and was not due to a change in channel expression. dCT titration using a wider range of RNA doses, while keeping the level of $Ca_V1.2\Delta1821$ constant, revealed that current inhibition by dCT was dose dependent, reaching a maximal 50–60% reduction at 2.5:1 dCT:channel RNA ratio (Fig. 4*B* and *C*).

The coexpressed dCT also produced the second expected effect, the depolarizing shift of the *G–V* curve, increasing the half-maximal activation voltage ($V_a$). However, this effect took place only at dCT:channel RNA ratios of $\geq$10:1 (Fig. 4*D* and *E*, Table 2), much higher than required for the maximal effect of dCT on $I_{Ba}$ amplitude. Evidently, the expression of the dCT protein further increased at RNA ratios higher than 5:1, as it produced an effect not seen at lower RNA doses. Thus, the saturation of dCT action on $I_{Ba}$ amplitude at a 2.5:1 dCT:channel ratio reflects a genuine maximal reduction in $I_{Ba}$ rather than a limitation of the oocyte's expression machinery (Oz *et al.* 2013). A similar distinction between the actions of dCT on $I_{Ba}$ amplitude and *G–V* curve was previously reported in mammalian tsA-201 cells (Hulme *et al.* 2006; Fuller *et al.* 2010); it may imply distinct molecular mechanisms for the two effects.

**Table 2. Boltzmann *I–V* fit parameters**

| | Mean | *n* | SEM | *t* test |
|---|---|---|---|---|
| $Ca_V1.2\Delta1821$ (1 ng) | | | | |
| $G_{max}$ | 45.9 | 25 | 4.2 | |
| $V_{rev}$ | 61.7 | 25 | 0.6 | |
| $V_a$ | −3.0 | 25 | 0.8 | |
| $K_a$ | 6.4 | 25 | 0.3 | |
| $Ca_V1.2\Delta1821$ (1 ng) + dCT (5 ng) | | | | |
| $G_{max}$ | 40.1 | 8 | 5.4 | |
| $V_{rev}$ | 62.9 | 8 | 1.0 | |
| $V_a$ | −2.3 | 8 | 2.9 | ns |
| $K_a$ | 6.7 | 8 | 0.6 | |
| $Ca_V1.2\Delta1821$ (1 ng) + dCT (10 ng) | | | | |
| $G_{max}$ | 24.0 | 9 | 2.0 | |
| $V_{rev}$ | 60.8 | 9 | 0.9 | |
| $V_a$ | 5.3 | 9 | 1.5 | *** |
| $K_a$ | 7.3 | 9 | 0.3 | |
| $Ca_V1.2\Delta1821$ (1 ng) + dCT (12.5 ng) | | | | |
| $G_{max}$ | 25.3 | 5 | 7.1 | |
| $V_{rev}$ | 70.4 | 5 | 5.7 | |
| $V_a$ | 6.8 | 5 | 2.5 | *** |
| $K_a$ | 7.5 | 5 | 1.0 | |

Statistical significance of the shift in half-maximal voltage ($V_a$) was determined using a *t* test, comparing to control group (no dCT expression). ***$P < 0.001$; ns, not significant.

It has been proposed that the cleaved dCT interacts with the proximal part of the CT in $Ca_V1.2\Delta1821$, and this interaction is essential for the proper regulation by PKA. By binding both $\alpha_{1C}$ (at the dCT) and PKA-RS, A-kinase-anchoring proteins (AKAPs) anchor PKA to $Ca_V1.2$. AKAP15 is an integral part of the $Ca_V1.2$ regulatory complex in cardiomyocytes (Catterall, 2015), and it was also found indispensable for PKA modulation of $Ca_V1.2$ reconstituted in HEK cells (Fuller *et al.* 2010, 2014). In contrast, our data suggest that in *Xenopus* oocytes, neither dCT nor AKAP15 are essential for the cAMP effect on $I_{Ba}$. We tested a range of dCT:channel and AKAP15:channel RNA ratios, but none have yielded a significant increase in cAMP effect on $I_{Ba}$ amplitude, compared with the channel alone (Fig. 5*A*).

We used the AKAP blocker Ht31 (Tröger *et al.* 2012; Dema *et al.* 2015) to test the possibility that endogenous AKAPs in the oocytes optimize the channel complex, obviating the need for exogenously expressed AKAP15. However, EGFP-fused Ht31 peptide (Lynch *et al.* 2005), which was well expressed in the oocytes as verified by confocal imaging, did not affect the cAMP-induced modulation of $Ca_V1.2\Delta1821$ (Fig. 5*B*). We conclude that, in *Xenopus* oocytes, reconstitution of PKA-CS regulation of $Ca_V1.2\Delta1821$ does not necessitate the presence of dCT or AKAP.

### cAMP modulation of $Ca_V1.2$ in *Xenopus* oocytes does not require the $\alpha_{1C}$ phosphorylation sites S1700 and T1704, or the $Ca_V\beta$ subunit

Serine-1700 and threonine-1704 were proposed to be crucial for PKA-induced $Ca_V1.2$ upregulation reconstituted in HEK cells (Fuller *et al.* 2010). We mutated these amino acids, separately or together, to alanines. These mutations did not attenuate the cAMP-dependent increase in $Ca_V1.2\Delta1821$ currents in oocytes (Fig. 6*A*).

The cardiac $Ca_V\beta$ subunit is phosphorylated *in vivo* following $\beta$-adrenergic stimulation (Haase *et al.* 1996). However, the role of phosphorylation of $Ca_V\beta$ in PKA enhancement of $Ca_V1.2$ is controversial (compare Bunemann *et al.* 1999 *vs.* Miriyala *et al.* 2008; Brandmayr *et al.* 2012; Minobe *et al.* 2014, and see Discussion). To test the involvement of $Ca_V\beta$ in our system, we prepared a construct, termed $\beta_{2b}$-4A, with four alanine mutations in PKA phosphorylation sites: serine 296 (Pankonien *et al.* 2012), serines 479 and 480 (Bunemann *et al.* 1999) and serine 576 (Viard *et al.* 2004). To exclude the involvement of any unrecognized phosphorylation sites in the CT of $Ca_V\beta$, another construct, truncated after amino acid 470, was prepared ($\beta_{2b}$-$CT_{trunc}$, Fig. 6*Ba*). The two constructs, $\beta_{2b}$-4A and $\beta_{2b}$-$CT_{trunc}$, were expressed in the oocyte along with the $\alpha_{1C}\Delta1821$ and $\alpha_2\delta$ subunits. The mutated $Ca_V\beta$ constructs did not attenuate the upregulation of $I_{Ba}$ following cAMP injection (Fig. 6*Bb* and *c*). Coexpression of AKAP15 and dCT constructs together with $\alpha_{1C}\Delta1821$ and $\alpha_2\delta$ and either $\beta_{2b}$-4A or $\beta_{2b}$-$CT_{trunc}$ did not significantly alter the cAMP effect on $I_{Ba}$ (Fig. 6*Bb*). Furthermore, $\alpha_{1C}\Delta1821$ coexpressed with the $\alpha_2\delta$ subunit only, without $Ca_V\beta$, showed cAMP modulation comparable to full subunit composition (Fig. 6*Bb* and *c*), indicating the redundancy of $Ca_V\beta$ in the cAMP modulation observed in *Xenopus* oocytes.

### N-terminus of $\alpha_{1C}$ is involved in cAMP-dependent PKA upregulation of $I_{Ba}$

The N-terminus (NT) of the cardiac $\alpha_{1C}$ encompasses an inhibitory module that regulates the channel's gating and is involved in $Ca_V1.2$ regulation by protein kinase C (PKC) (Shistik *et al.* 1998; Blumenstein *et al.* 2002; Weiss *et al.* 2012). We hypothesized that the NT may also be involved in PKA-dependent regulation of $Ca_V1.2$.

The cytosolic NT domain of the cardiac 'long-NT' (LNT) $\alpha_{1C}$ isoform is 154 amino acids long. The first 46 amino acids are encoded by the variable exon 1a and the rest by the constant exon 2 (Blumenstein *et al.* 2002; Dai *et al.* 2002; Pang *et al.* 2003). The inhibitory module comprises the first 20 a.a. of the initial 46 a.a. segment (Kanevsky & Dascal, 2006). In the smooth muscle 'short-NT' (SNT) isoform, also abundant in the brain (Biel *et al.* 1990; Koch *et al.* 1990; Snutch *et al.* 1991), the initial NT segment is 16 a.a. long and is encoded by exon 1 instead of 1a (Abernethy & Soldatov, 2002). The SNT $\alpha_{1C}$ does not contain an inhibitory module, and is not upregulated by PKC in *Xenopus* oocytes (Kanevsky & Dascal, 2006).

We compared the cAMP regulation of LNT and SNT $\alpha_{1C}$ isoforms, as well as a series of additional NT deletions and chimeric constructs, created on the template of the CT-truncated $\alpha_{1C}\Delta1821$ (numbering by LNT isoform) (Fig. 7*A*). cAMP injection into oocytes expressing the SNT $\alpha_{1C}\Delta1821$ isoform failed to enhance $I_{Ba}$, in contrast with the cardiac LNT $\alpha_{1C}\Delta1821$. The removal of most of the NT (LNT$\Delta$139) or of the inhibitory gating element alone (the first 20 a.a. of the LNT; LNT$\Delta$20) abolished the cAMP-dependent increase in $I_{Ba}$ (Fig. 7*A* and *B*). These results show that the initial 20 a.a. are essential for PKA regulation.

Importantly, addition of the inhibitory module (the first 20 a.a.) directly to the rest of NT encoded by exon 2 (by removing the last 26 amino acids of the exon 1a-encoded

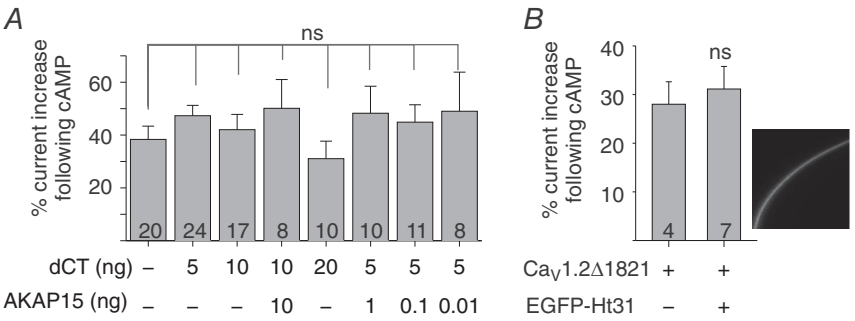


**Figure 5. dCT and AKAP15 do not alter the cAMP-dependent increase in $I_{Ba}$**
*A*, coexpression of dCT and AKAP15 does not alter the cAMP effect on $Ca_V1.2\Delta1821$. Oocytes were injected with 1 ng RNA of $Ca_V1.2\Delta1821$ and the indicated amounts of dCT and AKAP15 RNAs (summary of 5 experiments). *B*, coexpression of EGFP-labelled Ht31 (5 ng RNA) does not affect the regulation of $Ca_V1.2\Delta1821$ (1 ng RNA). Expression of EGFP-Ht31 was confirmed by measuring fluorescence in intact oocytes. Summary from 2 experiments is shown.

segment; LNTΔ20–46) fully preserved the cAMP-induced increase in $I_{Ba}$. Since this construct is the exact counterpart of the SNT $\alpha_{1C}$ except for the exchanged first short (20 or 16 a.a.) segment, we conclude that the first 20 a.a. of LNT are sufficient to support the PKA regulation in the context of $\alpha_{1C}$Δ1821.

Deletion of amino acids 2–5 in the inhibitory gating element of the LNT $\alpha_{1C}$Δ1821 eliminated the cAMP regulation (the LNTΔ5 construct), and greatly reduced it by ∼70% when both amino acids 2–5 and amino acids 20–46 of LNT $\alpha_{1C}$Δ1821 were deleted (LNTΔ5Δ20–46). Yet, adding amino acids 2–5 or amino acids 20–46 of the long NT to the SNT $\alpha_{1C}$Δ1821 did not render the SNT channel cAMP sensitive (chimeras 'SNT+a.a.2–5' and 'SNT+a.a.20–46'). Thus, amino acids 2–5 are essential but not sufficient for cAMP-dependent current upregulation.

The initial five amino acids of the LNT isoform are not fully conserved in different species. The a.a. sequence is MLRAL in rabbit, MLRAF in human, and MIRAF in mouse LNT $\alpha_{1C}$. We have tested for

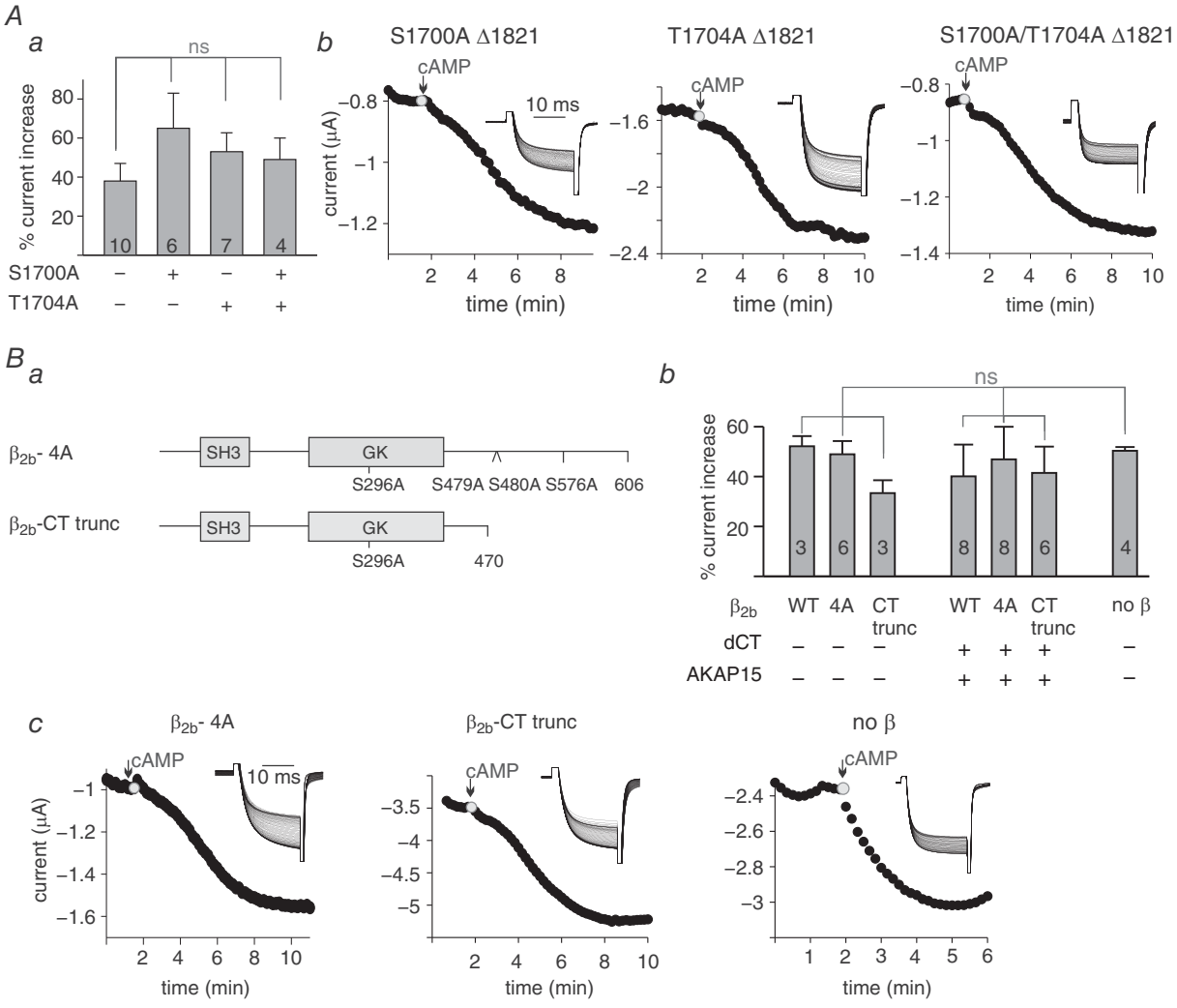


**Figure 6. cAMP-dependent $I_{Ba}$ upregulation is not affected by alanine mutations of S1700 and T1704 in $\alpha_{1C}$, or by the $Ca_V\beta$ subunit**
*A*, mutation S1700A, T1704A, or both to alanine do not alter the extent of cAMP upregulation of $Ca_V$Δ1821. *a*, summary of cAMP effect. $N = 2$ experiments. No statistical significance was found using one-way ANOVA test. *b*, representative time course charts of changes in $I_{Ba}$ following cAMP injection (200 $\mu$M). Insets in *b* show $I_{Ba}$ current traces in the same cell. *B*, the presence of $Ca_V\beta_{2b}$ subunit, or mutations in $Ca_V\beta_{2b}$ phosphorylation sites, do not alter cAMP regulation. *a*, an illustration of the constructs used. SH3 and GK are structural modules within the $\beta$ subunit. Numbers indicate amino acids. *b*, summary of % increase in $I_{Ba}$ per cell, following cAMP injection. Oocytes were injected with the following RNAs: 1 ng $\alpha_{1C}$Δ1821, $\alpha_2\delta1$ and either $Ca_V\beta_{2b}$-wild-type (WT), $Ca_V\beta_{2b}$-4A or $Ca_V\beta_{2b}$-$CT_{trunc}$, with or without 2.5 ng AKAP15 and 5 ng dCT. $\alpha_{1C}$Δ1821 RNA (7 ng) was injected when $Ca_V\beta_{2b}$ subunit was not present ($\alpha_{1C}$Δ1821 and $\alpha_2\delta1$ only). No statistical significance was found using one-way ANOVA test. *c*, representative time course charts of $I_{Ba}$ following cAMP injection (200 $\mu$M). Insets show current traces in the same cells.

potential species variability in PKA regulation using mouse $\alpha_{1C}$. Figure 8*A*–*C* shows that the dCT-truncated mouse $\alpha_{1C}\Delta1821$ was strongly regulated by the injection of purified PKA-CS, with an average increase of 126 ± 23% ($n$ = 12), which is almost 3-fold greater than the average ∼40% increase observed in rabbit $Ca_V1.2\Delta1821$ (compare to Fig. 1*G*). Deletion of amino acids 2–5 ($Ca_V1.2\Delta5\Delta1821$) strongly reduced, but did not eliminate, the PKA-CS effect: a residual 29 ± 12% increase was still present (Fig. 8*B* and *C*). Full-length $\alpha_{1C}$ with the deletion of amino acids 2–5 ($Ca_V1.2\Delta5$-full) was not upregulated by PKA-CS.

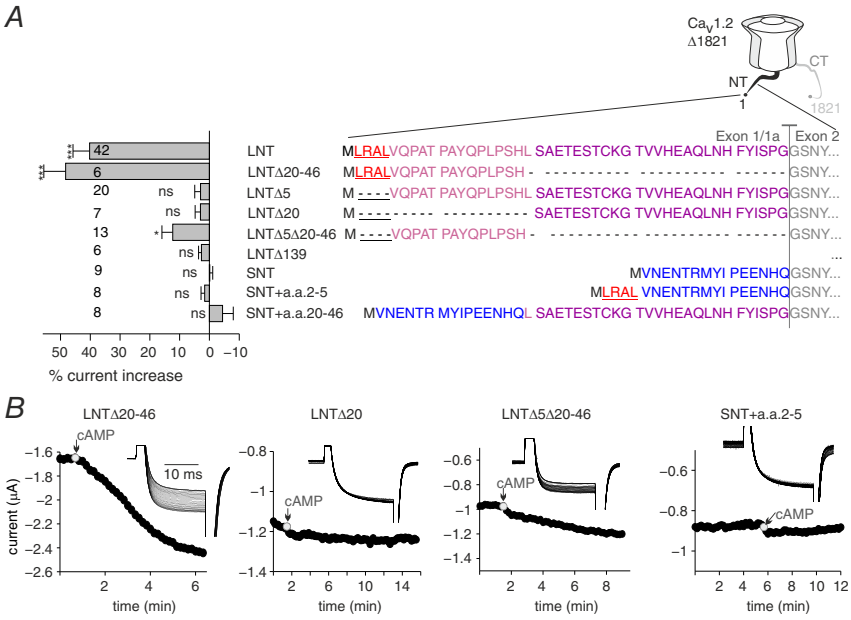


**Figure 7. The initial segment of the long-NT $\alpha_{1C}$ is essential for the $I_{Ba}$ enhancement by cAMP**
In this figure, LNT denotes the long-NT initial segment of $\alpha_{1C}$ encoded by exon 1a, and SNT denotes the short-NT encoded by exon 1. *A*, regulation of chimeras and N-terminal deletion mutants of rabbit $\alpha_{1C}\Delta1821$ by cAMP (200 $\mu$M). Right panel, the N-terminal amino acid sequences in $\alpha_{1C}\Delta1821$ constructs used. Δ139 is a deletion of most of the NT, including exon 1a and most of exon 2. Colour codes: blue, SNT initial segment (encoded by exon 1); the other colours correspond to segments of the initial segment of LNT; first methionine is in black. Left panel shows the summary of cAMP effect (11 experiments). Statistical significance was determined by paired *t* test. ***$P < 0.001$; *$P = 0.012$; ns, not significant. *B*, representative time course charts of changes in $I_{Ba}$ following cAMP injection in some of the constructs. Insets show current traces in the same cells.

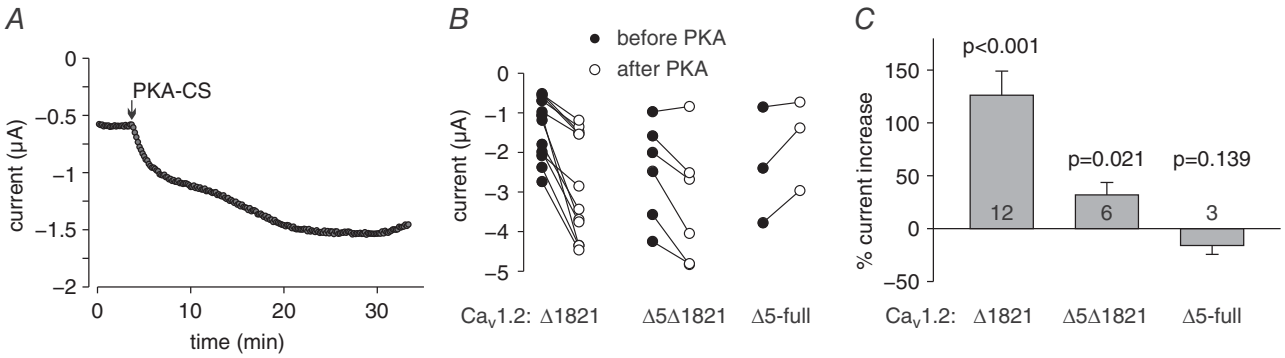


**Figure 8. PKA-CS enhances $I_{Ba}$ of dCT-truncated mouse $\alpha_{1C}$**
*A*, representative time course of $I_{Ba}$ changes in an oocyte expressing mouse $Ca_V1.2\Delta1821$. The experimental protocols were as with rabbit $Ca_V1.2$. PKA-CS was injected to a final concentration of about 4 $\mu$M. *B*, changes in $I_{Ba}$ in the three $\alpha_{1C}$ constructs used, caused by PKA-CS injection in individual oocytes. Filled circles show $I_{Ba}$ before PKA-CS injection; open circles show $I_{Ba}$ after full development of the effect, 10–25 min after injection. *C*, summary of two experiments. Statistical significance was calculated using paired *t* test.

In summary, the experiments with the N-terminal mutants of rabbit and mouse $\alpha_{1C}$ suggest that the initial 20 amino acid segment of $\alpha_{1C}$ uniquely present in the cardiac LNT isoform is essential for the cAMP and PKA regulation of $Ca_V1.2\Delta1821$ in *Xenopus* oocytes. This segment is also sufficient to render the channel sensitive to PKA when the rest of the channel (starting from the exon 2-encoded part) is present. The few partly conserved amino acids at the very beginning of this segment play an important role, and their removal eliminates >70% of the PKA effect.

## Discussion

The intensive effort to understand the mechanism of PKA regulation of $Ca_V1.2$ has not yet yielded a unifying mechanistic insight. Studies in genetically modified cardiomyocytes and mice refuted the proposed crucial roles of putative target phosphorylation sites in $\alpha_{1C}$ and $\beta$ subunits suggested by *in vitro* and heterologous studies. Disappointingly, so far, studies with genetically engineered animals also did not positively resolve the puzzle (Weiss *et al.* 2013; Hofmann *et al.* 2014; Catterall, 2015; Morrow & Marx, 2015). Nevertheless, one major mechanistic insight is shared by most published works: the involvement of the distal C-terminus (dCT) of $\alpha_{1C}$, and the importance of its naturally occurring post-translational truncation (Fuller *et al.* 2010; Domes *et al.* 2011; Fu *et al.* 2011). With this in mind, in this work we have re-examined the possibility of utilizing the *Xenopus* oocyte to search for additional unknown components of the PKA regulatory mechanism.

We succeeded to reconstitute the cAMP/PKA regulation of the dCT-truncated $Ca_V1.2$ in *Xenopus* oocytes, a task which previously failed with the full-length, non-truncated $\alpha_{1C}$. A reproducible whole-cell current increase of $Ca_V1.2$ from two species, rabbit and mouse, has been observed in single oocytes following intracellular application (injection) of cAMP or PKA-CS. Although truncation of the dCT was essential, the presence of the clipped dCT or AKAP15 was not. The cAMP/PKA effect did not involve the phosphorylation sites S1700 and T1704 in $\alpha_{1C}$, or the $\beta$ subunit of the channel, in line with recent animal model studies (Brandmayr *et al.* 2012; Yang *et al.* 2013). These findings pave the way for the further use of this versatile and easily manipulated heterologous system to pinpoint the molecular determinants of PKA modulation within $Ca_V1.2$. To this end, using a standard mutagenesis approach, we identified the initial segment of the cardiac LNT isoform of $\alpha_{1C}$ as a previously unrecognized requisite structural element involved in PKA regulation. This finding raises the possibility that PKA is acting, in part, by affecting the hypothesized synergistic regulation of $Ca_V1.2$ by a scaffold formed by cytosolic N- and C-termini of $\alpha_{1C}$ (Ivanina *et al.* 2000; Dick *et al.* 2008; Benmocha Guggenheimer *et al.* 2016). We further propose that the new mechanism may be one of two or more complementary pathways of PKA regulation of $Ca_V1.2$ in the heart.

### cAMP regulation of $Ca_V1.2\Delta1821$ is PKA specific and requires dissociation of PKA subunits

Our results unequivocally demonstrate that $Ca_V1.2$, with its main $\alpha_{1C}$ subunit truncated approximately at the same site as in the cardiomyocytes, is specifically regulated by PKA-CS in *Xenopus* oocytes. The use of *Xenopus* oocytes allowed several advantages for the study of L-type $Ca^{2+}$ channels. First, we used two-electrode voltage clamp with sharp electrodes and injected cAMP, PKA-CS and other substances directly into the cell. This method avoids the dilution of the cytosol and the accompanying rundown of the channel's current inherent in whole-cell patch clamp (Weiss *et al.* 2013) used in mammalian cell models. Further, the injection technique allowed $Ba^{2+}$ currents to be measured via $Ca_V1.2$ before and after cAMP/PKA addition in the same cell, thus allowing sensitive detection of even small changes in whole-cell currents. Finally, oocytes are highly suitable for titrated expression of proteins, due to precise control of the amount of injected RNA (e.g. Yakubovich *et al.* 2015).

As positive control for the functional effects of cAMP and PKA-CS in *Xenopus* oocytes, we monitored PKA-dependent changes in $Cl^-$ currents of the CFTR channel, a well-characterized PKA effector (Gadsby & Nairn, 1999). CFTR's $Cl^-$ currents were greatly enhanced by the injection of cAMP or PKA-CS, and specificity of PKA regulation was confirmed through inhibition by the inactive cAMP analogue Rp-cAMPS and the highly specific PKA inhibitory protein PKI (Fig. 3). By using the same concentrations of Rp-cAMPS and PKI, we confirmed the inhibition of cAMP-dependent $Ca_V1.2\Delta1821$ upregulation (Fig. 3*C* and *D*). Thus, the regulation of $Ca_V1.2$ is PKA specific.

The results also strongly suggest that the observed cAMP regulation involved the classical mechanism of dissociation of PKA-CS from the PKA-RS which, in the absence of cAMP, is strongly associated with, and inhibits the activity of, PKA-CS (Taylor *et al.* 2012). This is supported by the enhancing action of purified PKA-CS and the strong blocking effect of Rp-cAMPS, which acts mainly by preventing the dissociation of the PKA holoenzyme (Anand *et al.* 2010).

### The discrepancies with previous reports

The open questions regarding the mechanism of PKA regulation of $Ca_V1.2$ have been thoroughly reviewed in the past (Weiss *et al.* 2013; Hofmann *et al.* 2014). There were disagreements on experimental findings even in the same expression system such as HEK cells or their derivative, the tsA cell line (for example, Gao *et al.* 1997

*vs.* Fuller *et al.* 2010 *vs.* Zong *et al.* 1995). At the same time, there was a general consensus on the absence of PKA upregulation of full-length (untruncated) $Ca_V1.2$ across different heterologous systems (Perez Reyes *et al.* 1994; Singer-Lahat *et al.* 1994; Zong *et al.* 1995), as confirmed here (Fig. 1). In the following we discuss how some of the discrepancies can be reconciled considering potential differences between cell types and the importance of colocalization of $Ca_V1.2$ and PKA in cardiomyocytes. Our emphasis is on the recent extensive studies of Fuller *et al.* done in tsA cells (Fuller *et al.* 2010, 2014). The two major points of disagreement with the latter work are the requirement for a simultaneous presence of dCT and AKAP, and the importance of phosphorylation of S1700 and T1704.

### Truncation of dCT is crucial for PKA regulation of $Ca_V1.2$, but the dCT and AKAP15 are not essential

Our study agrees with that of Fuller *et al.* (2010) in one central aspect: as in tsA cells, in the oocytes the truncation of dCT of $\alpha_{1C}$ is essential for PKA-induced regulation of $Ca_V1.2$. In the oocytes, current via the dCT-truncated channel, $Ca_V1.2\Delta1821$, was increased by ∼40% (rabbit) or ∼130% (mouse $\alpha_{1C}$) by the injected cAMP or purified PKA-CS, whereas the full-length channel was not regulated (Figs 1 and 8).

In our hands, the PKA-induced increase in current amplitude of $Ca_V1.2\Delta1821$ in the oocytes did not require the clipped dCT, and cAMP-induced increase in channel current was not altered in the very wide dCT titration range used here. The dCT was synthesized and expressed as a separate protein in the oocytes, and interacted with $\alpha_{1C}$, because it produced the two well-documented effects on the truncated $Ca_V1.2$: a reduction in whole-cell current (without any change in surface expression of $\alpha_{1C}$) and, at higher expression levels of the dCT, a depolarized shift in channel activation (Fig. 4) (Hulme *et al.* 2006; Fuller *et al.* 2010).

Further, titration of AKAP15 had no additional effects on cAMP-dependent upregulation of $Ca_V1.2\Delta1821$ (Fig. 5). We cannot completely rule out a role for an endogenous oocyte AKAP. However, two lines of evidence argue against this possibility. First, Ht31, a peptide that inhibits interaction of AKAPs with the PKA regulatory subunit and disrupts PKA anchoring by AKAPs, did not influence the effect of cAMP. Second, the dCT, which anchors both AKAP15 and AKAP79 to $\alpha_{1C}$ (Catterall, 2015), was not needed. The expendability of AKAP does not contradict *in vivo* studies, because PKA regulation of cardiac $Ca_V1.2$ is preserved after knockout of AKAP15 and AKAP79/150 in mice (Nichols *et al.* 2010; Jones *et al.* 2012).

The above results are at odds with the reported necessity for the simultaneous presence of dCT and AKAP in the tsA-201 cell line, where cAMP levels were elevated with the adenylyl cyclase activator, forskolin (Fuller *et al.* 2010, 2014). Nevertheless, a comparative analysis of oocyte and tsA-201 data offers a plausible way to reconcile among them, and to explain why forskolin cannot regulate $Ca_V1.2\Delta1821$ in the absence of dCT and AKAP in tsA-201 cells. The logic is as follows:

(1) It is widely agreed that AKAP is needed only for anchoring the PKA close to the channel; it is not implicated in the end-point of regulation itself (Catterall, 2015).
(2) AKAP15 is anchored to $Ca_V1.2$ via the dCT of $\alpha_{1C}$. The dCT is essential to allow close apposition of PKA and $Ca_V1.2$.
(3) In tsA cells, no regulation of $\alpha_{1C}\Delta1800$ by forskolin is seen without AKAP15, even when dCT is present. It follows that forskolin-induced activation of endogenous PKA in tsA cells is insufficient for $Ca_V1.2$ regulation without the juxtaposition of PKA and $\alpha_{1C}$ via AKAP.
(4) In summary, we contend that the Fuller *et al.* (2010) data show the necessity of the dCT for anchoring of PKA to $\alpha_{1C}$ via AKAP, but do not necessarily implicate the dCT in the end-point regulation mechanism itself.
(5) In the oocytes, we are providing the injected cell with ample cAMP or purified PKA-CS, which obviously bypasses the need for PKA anchoring near the channel, since we see a bold regulation without adding AKAPs.

In partial support of this hypothesis, a previous study in HEK cells reported upregulation of $Ca_V1.2$ by infused PKA-CS in the absence of dCT or AKAP, when $\alpha_{1C}$ was truncated at amino acid 1905 (Bunemann *et al.* 1999).

We conclude that, on the factual level, the dispensability of dCT and AKAP15 in oocytes does not necessarily contradict the phenomena observed in HEK/tsA cells. However, on the mechanistic level, our findings do not support the elegant mechanism proposed by Catterall and colleagues (Fuller *et al.* 2010), where phosphorylation of S1700 removes a dCT-imposed channel inhibition. This is because in the oocytes PKA regulates $Ca_V1.2\Delta1821$ in the absence of dCT. Notably, Beam *et al.* arrived at a similar conclusion for the skeletal muscle $Ca_V1.1$, where dCT is truncated but does not associate with or modulate the channel, yet the channel is regulated by PKA, though rather modestly (Ohrtman *et al.* 2015). The expendability of dCT does not undermine the importance of the rest of the CT for PKA regulation. For instance, the fact that in the context of full-length $\alpha_{1C}$ the dCT impairs the effect of PKA hints upon a folding effect of the dCT, possibly by its binding to proximal CT.

### Prominent phosphorylation sites in $\alpha_{1C}$ and $\beta$ subunits are not involved in PKA regulation of the truncated $Ca_V1.2$ in *Xenopus* oocytes

On the basis of experiments in HEK cells, phosphorylation of C-terminal serines in the $Ca_V\beta$ subunit has been proposed to play a major role in PKA regulation of $Ca_V1.2$ (Bunemann *et al.* 1999). In our oocyte system, cAMP similarly regulated $Ca_V1.2\Delta1821$ with or without the coexpression of the $Ca_V\beta$ subunit (Fig. 6). Involvement of the oocytes' endogenous $Ca_V\beta_3$ subunit (Tareilus *et al.* 1997) is unlikely, since $Ca_V\beta$ subunit overexpression greatly changes the amplitude and activation of the expressed $Ca^{2+}$ channels, suggesting that most of them are not pre-associated with the endogenous $Ca_V\beta$ (Canti *et al.* 2001). By mutagenesis, we rule out the involvement of $Ca_V\beta$ phosphorylation sites 296, 479, 480 and 576. Our results corroborate the previous expression studies in the mammalian BHK cell line (Minobe *et al.* 2014) and in cardiomyocytes (Miriyala *et al.* 2008), and in genetically engineered mice, where truncation of $Ca_V\beta$ at position 450 (a.a. count according to rabbit $Ca_V\beta_{2b}$ isoform used here) did not affect $\beta$-adrenergic $I_{Ca}$ upregulation (Brandmayr *et al.* 2012). Taken together, our data suggest that phosphorylation of the $Ca_V\beta$ subunit is not involved in the PKA effect on $Ca_V1.2\Delta1821$ reconstituted in *Xenopus* oocytes.

The role of PKA phosphorylation sites in the $\alpha_{1C}$ subunit has been highly controversial. The involvement of S1928, a major PKA phosphorylation site previously thought to mediate the PKA effect (reviewed in Weiss *et al.* 2013), was ruled out by functional studies in genetically engineered cardiomyocytes and mice (Ganesan *et al.* 2006; Lemke *et al.* 2008). Phosphorylation of this serine has recently been found crucial for uncoupling of the direct interaction between $\beta$2AR and $Ca_V1.2$ (Patriarchi *et al.* 2016). In our system, S1928 is irrelevant because it is located in the dCT which is not needed for the PKA regulation of $Ca_V1.2\Delta1821$.

Phosphorylation of two additional sites located in the proximal CT, S1700 and T1704 (Norman & Leach, 1994; Fuller *et al.* 2010), has been proposed to mediate the effect of PKA in $Ca_V1.2$ by weakening the interaction of dCT with a proximal CT-acceptor site, PCRD (Fuller *et al.* 2010; Catterall, 2015). However, more recently it has been proposed that T1704 (which is not a PKA but rather a casein kinase site; Fuller *et al.* 2010) is probably not involved in PKA regulation but may contribute to the basal activity of the channel (Fu *et al.* 2014). In our case, alanine mutations of S1700 and T1704 in $Ca_V1.2\Delta1821$ expressed in oocytes did not reduce the sensitivity to cAMP (Fig. 6*A*). This result correlates with the reported lack of effect of mutating these residues in a mouse model with inducible expression of the mutated $\alpha_{1C}$ (Yang *et al.* 2013). Nevertheless, studies of a different mouse model show that mutation of S1700 alters the regulation of $Ca_V1.2$ function, and PKA and $Ca^{2+}$ handling in mouse heart (Fu *et al.* 2014; Yang *et al.* 2016), although the exact role of phosphorylation of S1700 in overall PKA effect on $Ca_V1.2$ itself may need further clarification. In heterologous systems, diverse cellular properties certainly may play a role, such that the fractional contribution of phosphorylation of S1700 (possibly followed by separation of the dCT) is prominent in tsA cells but not in the oocytes. Resolving this conundrum will require further study.

### A novel mechanism involving the N-terminus of $\alpha_{1C}$

We have utilized the newly developed experimental approach to look for additional molecular determinants of PKA regulation in $\alpha_{1C}$. The NT is an important regulatory domain that controls the channel's open probability, trafficking and inactivation (Shistik *et al.* 1998; Ivanina *et al.* 2000; Kanevsky & Dascal, 2006; Dick *et al.* 2008; Thomsen *et al.* 2009; Simms *et al.* 2015). Previously we have shown that PKC regulation of $Ca_V1.2$ expressed in *Xenopus* oocytes is controlled by the NT inhibitory gating element through a mechanism that does not involve phosphorylation of the gating element itself, and does not require the C-terminal truncation of $\alpha_{1C}$ (Shistik *et al.* 1998, 1999; Blumenstein *et al.* 2002). This gating element comprises the first 20 amino acids, out of the 46 amino acids encoded by exon 1a, of the 'cardiac' LNT isoform of $\alpha_{1C}$ (Kanevsky & Dascal, 2006). Here we show that this 20 a.a. segment is essential for PKA regulation of cardiac $Ca_V1.2$ reconstituted in *Xenopus* oocytes (Fig. 7). This 20 a.a. segment is also sufficient for rendering the $Ca_V1.2\Delta1821$ sensitive to PKA when added to the rest of $\alpha_{1C}$ starting from the part of NT encoded by the obligatory exon 2. The partially homologous 16 a.a. segment of the short NT isoform, encoded by exon 1, does not carry this property, implying the presence and importance of a highly specific element or structure within the 20 amino acids of the cardiac LNT isoform of $\alpha_{1C}$. The first five amino acids may be part of this element, since the removal of this segment reduced the PKA effect by $>$70% (Figs 7 and 8). Nevertheless, the addition of five initial amino acids did not confer PKA regulation upon the SNT $Ca_V1.2\Delta1831$. Thus, the first five amino acids are important, but not sufficient for the PKA effect. We have previously proposed that NT and CT may act together as one functional unit (an 'NT–CT scaffold') to regulate the channel's gating (Ivanina *et al.* 2000), and demonstrated a direct interaction (binding) between the NT and CT of $\alpha_{1C}$ (Benmocha Guggenheimer *et al.* 2016). We hypothesize that the 'NT–CT scaffold' participates in PKA regulation of $Ca_V1.2$ gating.

### One or more mechanisms of $Ca_V1.2$ regulation by PKA?

The regulation of $\alpha_{1C}$ truncated at 1821 is at odds with two reports in genetically engineered cardiomyocytes and

mice, where truncation of $\alpha_{1C}$ at a.a. 1905 or 1796 produced channels with low or no sensitivity to $\beta$-AR and PKA activators (Ganesan *et al.* 2006; Fu *et al.* 2011). Notably, however, in genetically engineered mice the expression of C-terminally truncated channels is severely reduced, and localized expression of AKAP15 is also reduced (Domes *et al.* 2011; Fu *et al.* 2011). In cardiomyocytes, the truncated channels were expressed ectopically (Ganesan *et al.* 2006) and might have not been localized in the correct cellular compartment. The $\beta$2AR is also expected to uncouple from $Ca_V1.2$ in the absence of dCT of $\alpha_{1C}$ (Patriarchi *et al.* 2016). Localization is crucial in cardiomyocytes (Balijepalli *et al.* 2006; McConnachie *et al.* 2006). Therefore, one reason for the observed lack of regulation could be insufficient anchoring of components of the $\beta$-AR–PKA pathway in the vicinity of the channel, or insufficient increase in cAMP concentration at the channel's location. Under our conditions, we saturated the channel with an excess of injected cAMP or PKA-CS to achieve the maximal possible response. Another (or an additional) possibility is that the mechanism described here is one of two or several redundant PKA regulation pathways (Weiss *et al.* 2013) that, together, produce the strong regulation of the channel observed in cardiomyocytes.

In this respect, it is interesting to compare the extent of increase in $Ca^{2+}$ channel currents in cardiac cells of the relevant species with that seen in our system. In isolated rabbit cardiomyocytes, the increase in the current above basal level varies from 50 to 130% with isoproterenol (isoprenaline) or forskolin (Han *et al.* 1994; Shannon *et al.* 1995; Matsuda *et al.* 1996; Ginsburg & Bers, 2004). In the mouse, the current is increased by $\beta$-AR activation anywhere from 45 to 200% above the basal level (Sako *et al.* 1997; Heubach *et al.* 1999; Lemke *et al.* 2008), 50–100% by forskolin, and up to 200% above baseline by purified PKA-CS (Maltsev *et al.* 1999). The 40% increase caused by cAMP of PKA-CS with rabbit $Ca_V1.2\Delta1821$ and the ∼130% increase caused by PKA-CS with mouse $Ca_V1.2\Delta1821$ are comparable to the lower or medium values from this (admittedly incomplete) list. It is therefore plausible that the observed effect might account for only a part of the total PKA regulation. As discussed above, phosphorylation of S1700 may be the other molecular event contributing to the total regulation.

In summary, we propose that several PKA regulation pathways may exist under physiological conditions. The regulation of the truncated $Ca_V1.2$ observed in the *Xenopus* oocyte expression system appears to be a very basic one, inherent to $\alpha_{1C}$. It does not require the $\beta$ subunit, AKAPs or the dCT (the latter actually hinders the regulation in the context of a full-length, non-truncated $\alpha_{1C}$). This expression system proved appropriate to observe the specific PKA regulation of the C-terminally truncated channel and to uncover the potential role of the NT. In native heart cells, more layers of complexity are added by localization and compartmentalization, anchoring, and regulatory proteins and other factors. The basic regulation of the truncated $\alpha_{1C}$ may coexist with AKAP15 and dCT-dependent regulation (Catterall, 2015), or some as yet unknown regulation of the full-length (non-truncated) $\alpha_{1C}$, which may differentially contribute to the total PKA effect.

## References


Abernethy DR & Soldatov NM (2002). Structure-functional diversity of human L-type $Ca^{2+}$ channel: perspectives for new pharmacological targets. *J Pharmacol Exp Ther* **300**, 724–728.

Anand GS, Krishnamurthy S, Bishnoi T, Kornev A, Taylor SS & Johnson DA (2010). Cyclic AMP- and ($R_p$)-cAMPS-induced conformational changes in a complex of the catalytic and regulatory (RI$\alpha$) subunits of cyclic AMP-dependent protein kinase. *Mol Cell Proteomics* **9**, 2225–2237.

Balijepalli RC, Foell JD, Hall DD, Hell JW & Kamp TJ (2006). Localization of cardiac L-type $Ca^{2+}$ channels to a caveolar macromolecular signaling complex is required for $\beta_2$-adrenergic regulation. *Proc Natl Acad Sci USA* **103**, 7500–7505.

Bear CE, Duguay F, Naismith AL, Kartner N, Hanrahan JW & Riordan JR (1991). $Cl^-$ channel activity in *Xenopus* oocytes expressing the cystic fibrosis gene. *J Biol Chem* **266**, 19142–19145.

Beavo JA, Bechtel PJ & Krebs EG (1974). Activation of protein kinase by physiological concentrations of cyclic AMP. *Proc Natl Acad Sci USA* **71**, 3580–3583.

Benmocha Guggenheimer A, Almagor L, Tsemakhovich V, Tripathy DR, Hirsch JA & Dascal N (2016). Interactions between N and C termini of $\alpha_{1C}$ subunit regulate inactivation of $Ca_V1.2$ L-type $Ca^{2+}$ channel. *Channels* **10**, 55–68.

Bers DM (2000). Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* **87**, 275–281.

Bers DM (2008). Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol* **70**, 23–49.

Biel M, Ruth P, Bosse E, Hullin R, Stuhmer W, Flockerzi V & Hofmann F (1990). Primary structure and functional expression of a high voltage activated calcium channel from rabbit lung. *FEBS Lett* **269**, 409–412.

Blumenstein Y, Kanevsky N, Sahar G, Barzilai R, Ivanina T & Dascal N (2002). A novel long-N-terminus isoform of human L-type $Ca^{2+}$ channel is up-regulated by protein kinase C. *J Biol Chem* **277**, 3419–3423.

Brandmayr J, Poomvanicha M, Domes K, Ding J, Blaich A, Wegener JW, Moosmang S & Hofmann F (2012). Deletion of the C-terminal phosphorylation sites in the cardiac $\beta$-subunit does not affect the basic $\beta$-adrenergic response of the heart and the $Ca_v1.2$ channel. *J Biol Chem* **287**, 22584–22592.

Bunemann M, Gerhardstein BL, Gao T & Hosey MM (1999). Functional regulation of L-type calcium channels via protein kinase A- mediated phosphorylation of the $\beta$2 subunit. *J Biol Chem* **274**, 33851–33854.

Cachelin AB, de Peyer JE, Kokubun S & Reuter H (1983). $Ca^{2+}$ channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature* **304**, 462–464.

Campiglio M & Flucher BE (2015). The role of auxiliary subunits for the functional diversity of voltage-gated calcium channels. *J Cell Physiol* **230**, 2019–2031.

Canti C, Davies A, Berrow NS, Butcher AJ, Page KM & Dolphin AC (2001). Evidence for two concentration-dependent processes for $\beta$-subunit effects on $\alpha_{1B}$ calcium channels. *Biophys J* **81**, 1439–1451.

Carr DW, Hausken ZE, Fraser ID, Stofko-Hahn RE & Scott JD (1992). Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. *J Biol Chem* **267**, 13376–13382.

Catterall WA (2000). Structure and regulation of voltage-gated $Ca^{2+}$ channels. *Annu Rev Cell Dev Biol* **16**, 521–555.

Catterall WA (2015). Regulation of cardiac calcium channels in the fight-or-flight response. *Curr Mol Pharmacol* **8**, 12–21.

Charnet P, Lory P, Bourinet E, Collin T & Nargeot J (1995). cAMP-dependent phosphorylation of the cardiac L-type Ca channel: a missing link? *Biochimie* **77**, 957–962.

Crump SM, Andres DA, Sievert G & Satin J (2013). The cardiac L-type calcium channel distal carboxy terminus autoinhibition is regulated by calcium. *Am J Physiol Heart Circ Physiol* **304**, H455–H464.

Csanady L, Seto-Young D, Chan KW, Cenciarelli C, Angel BB, Qin J, McLachlin DT, Krutchinsky AN, Chait BT, Nairn AC & Gadsby DC (2005). Preferential phosphorylation of R-domain serine 768 dampens activation of CFTR channels by PKA. *J Gen Physiol* **125**, 171–186.

Dai B, Saada N, Echetebu C, Dettbarn C & Palade P (2002). A new promoter for $\alpha$1C subunit of human L-type cardiac calcium channel $Ca_v1.2$. *Biochem Biophys Res Commun* **296**, 429–433.

Dascal N & Lotan I (1992). Expression of exogenous ion channels and neurotransmitter receptors in RNA-injected *Xenopus* oocytes. In *Protocols in Molecular Neurobiology*, vol. **13**., ed. Longstaff A & Revest P, pp. 205–225. Humana Press, Totowa, NJ, USA.

Dema A, Perets E, Schulz MS, Deak VA & Klussmann E (2015). Pharmacological targeting of AKAP-directed compartmentalized cAMP signalling. *Cell Signal* **27**, 2474–2487.

Dick IE, Tadross MR, Liang H, Tay LH, Yang W & Yue DT (2008). A modular switch for spatial $Ca^{2+}$ selectivity in the calmodulin regulation of $Ca_v$ channels. *Nature* **451**, 830–834.

Dolphin AC (2012). Calcium channel auxiliary $\alpha 2\delta$ and $\beta$ subunits: trafficking and one step beyond. *Nat Rev Neurosci* **13**, 542–555.

Domes K, Ding J, Lemke T, Blaich A, Wegener JW, Brandmayr J, Moosmang S & Hofmann F (2011). Truncation of murine $Ca_v1.2$ at Asp-1904 results in heart failure after birth. *J Biol Chem* **286**, 33863–33871.

Florea VG & Cohn JN (2014). The autonomic nervous system and heart failure. *Circ Res* **114**, 1815–1826.

Fraser ID, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marrion NV & Scott JD (1998). A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. *EMBO J* **17**, 2261–2272.

Frohnwieser B, Chen LQ, Schreibmayer W & Kallen RG (1997). Modulation of the human cardiac sodium channel alpha-subunit by cAMP-dependent protein kinase and the responsible sequence domain. *J Physiol* **498**, 309–318.

Fu Y, Westenbroek RE, Scheuer T & Catterall WA (2013). Phosphorylation sites required for regulation of cardiac calcium channels in the fight-or-flight response. *Proc Natl Acad Sci USA* **110**, 19621–19626.

Fu Y, Westenbroek RE, Scheuer T & Catterall WA (2014). Basal and $\beta$-adrenergic regulation of the cardiac calcium channel $Ca_V1.2$ requires phosphorylation of serine 1700. *Proc Natl Acad Sci USA* **111**, 16598–16603.

Fu Y, Westenbroek RE, Yu FH, Clark JP, Marshall MR, Scheuer T & Catterall WA (2011). Deletion of the distal C terminus of $Ca_V1.2$ channels leads to loss of $\beta$-adrenergic regulation and heart failure in vivo. *J Biol Chem* **286**, 12617–12626.

Fuller MD, Emrick MA, Sadilek M, Scheuer T & Catterall WA (2010). Molecular mechanism of calcium channel regulation in the fight-or-flight response. *Sci Signal* **3**, ra70.

Fuller MD, Fu Y, Scheuer T & Catterall WA (2014). Differential regulation of $Ca_V1.2$ channels by cAMP-dependent protein kinase bound to A-kinase anchoring proteins 15 and 79/150. *J Gen Physiol* **143**, 315–324.

Gadsby DC & Nairn AC (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol Rev* **79**, S77–S107.

Ganesan AN, Maack C, Johns DC, Sidor A & O'Rourke B (2006). $\beta$-Adrenergic stimulation of L-type $Ca^{2+}$ channels in cardiac myocytes requires the distal carboxyl terminus of $\alpha_{1C}$ but not serine 1928. *Circ Res* **98**, e11–18.

Gao T, Cuadra AE, Ma H, Bunemann M, Gerhardstein BL, Cheng T, Ten Eick R & Hosey MM (2001). C-terminal fragments of the $\alpha_{1C}$ ($Ca_v1.2$) subunit associate with and regulate L-type calcium channels containing C-terminally truncated $\alpha_{1C}$ subunits. *J Biol Chem* **276**, 21089–21097.

Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD & Hosey MM (1997). cAMP-dependent regulation of cardiac L-type $Ca^{2+}$ channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* **19**, 185–196.

Gerhardstein BL, Gao T, Bünemann M, Puri TS, Adair A, Ma H & Hosey MM (2000). Proteolytic processing of the C terminus of the $\alpha$1C subunit of L-type calcium channels and the role of a proline-rich domain in membrane tethering of proteolytic fragments. *J Biol Chem* **275**, 8556–8563.

Ginsburg KS & Bers DM (2004). Modulation of excitation-contraction coupling by isoproterenol in cardiomyocytes with controlled SR $Ca^{2+}$ load and $Ca^{2+}$ current trigger. *J Physiol* **556**, 463–480.

Haase H, Bartel S, Karczewski P, Morano I & Krause EG (1996). In-vivo phosphorylation of the cardiac L-type calcium channel beta-subunit in response to catecholamines. *Mol Cell Biochem* **163–164**, 99–106.

Han J, Leem C, So I, Kim E, Hong S, Ho W, Sung H & Earm YE (1994). Effects of thyroid hormone on the calcium current and isoprenaline-induced background current in rabbit ventricular myocytes. *J Mol Cell Cardiol* **26**, 925–935.

Hartzell HC, Hirayama Y & Petit-Jacques J (1995). Effects of protein phosphatase and kinase inhibitors on the cardiac L-type Ca current suggest two sites are phosphorylated by protein kinase A and another protein kinase. *J Gen Physiol* **106**, 393–414.

Hartzell HC, Mery PF, Fischmeister R & Szabo G (1991). Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. *Nature* **351**, 573–576.

Heubach JF, Trebeß I, Wettwer E, Himmel HM, Michel MC, Kaumann AJ, Koch WJ, Harding SE & Ravens U (1999). L-type calcium current and contractility in ventricular myocytes from mice overexpressing the cardiac $\beta$2-adrenoceptor. *Cardiovasc Res* **42**, 173–182.

Hirayama Y & Hartzell HC (1997). Effects of protein phosphatase and kinase inhibitors on $Ca^{2+}$ and $Cl^{-}$ currents in guinea pig ventricular myocytes. *Mol Pharmacol* **52**, 725–734.

Hofmann F, Flockerzi V, Kahl S & Wegener JW (2014). L-type $Ca_V1.2$ calcium channels: from in vitro findings to in vivo function. *Physiol Rev* **94**, 303–326.

Hulme JT, Konoki K, Lin TW-C, Gritsenko MA, Camp DG, Bigelow DJ & Catterall WA (2005). Sites of proteolytic processing and noncovalent association of the distal C-terminal domain of $Ca_V1.1$ channels in skeletal muscle. *Proc Natl Acad Sci USA* **102**, 5274–5279.

Hulme JT, Yarov-Yarovoy V, Lin TW-C, Scheuer T & Catterall WA (2006). Autoinhibitory control of the $Ca_V1.2$ channel by its proteolytically processed distal C-terminal domain. *J Physiol* **576**, 87–102.

Ivanina T, Blumenstein Y, Shistik E, Barzilai R & Dascal N (2000). Modulation of L-type $Ca^{2+}$ channels by G$\beta\gamma$ and calmodulin via interactions with N- and C-termini of $\alpha_{1C}$. *J Biol Chem* **275**, 39846–39854.

Jones BW, Brunet S, Gilbert ML, Nichols CB, Su T, Westenbroek RE, Scott JD, Catterall WA & McKnight GS (2012). Cardiomyocytes from AKAP7 knockout mice respond normally to adrenergic stimulation. *Proc Natl Acad Sci USA* **109**, 17099–17104.

Kahanovitch U, Tsemakhovich V, Berlin S, Rubinstein M, Styr B, Castel R, Peleg S, Tabak G, Dessauer CW, Ivanina T & Dascal N (2014). Recruitment of G$\beta\gamma$ controls the basal activity of GIRK channels: crucial role of distal C-terminus of GIRK1. *J Physiol* **592**, 5373–5390.

Kameyama M, Hescheler J, Hofmann F & Trautwein W (1986). Modulation of Ca current during the phosphorylation cycle in the guinea pig heart. *Pflugers Arch* **407**, 123–128.

Kanevsky N & Dascal N (2006). Regulation of maximal open probability is a separable function of $Ca_v\beta$ subunit in L-type $Ca^{2+}$ channel, dependent on $NH_2$ terminus of $\alpha_{1C}$ ($Ca_v1.2\alpha$). *J Gen Physiol* **128**, 15–36.

Kim C, Cheng CY, Saldanha SA & Taylor SS (2007). PKA-I holoenzyme structure reveals a mechanism for cAMP-dependent activation. *Cell* **130**, 1032–1043.

Kim C, Xuong NH & Taylor SS (2005). Crystal structure of a complex between the catalytic and regulatory (RI$\alpha$) subunits of PKA. *Science* **307**, 690–696.

Klockner U, Mikala G, Eisfeld J, Iles DE, Strobeck M, Mershon JL, Schwartz A & Varadi G (1997). Properties of three COOH-terminal splice variants of a human cardiac L-type $Ca^{2+}$-channel $\alpha$1-subunit. *Am J Physiol* **272**, H1372–H1381.

Knighton DR, Zheng JH, Ten Eyck LF, Xuong NH, Taylor SS & Sowadski JM (1991). Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 414–420.

Koch WJ, Ellinor PT & Schwartz A (1990). cDNA cloning of a dihydropyridine-sensitive calcium channel from rat aorta. Evidence for the existence of alternatively spliced forms. *J Biol Chem* **265**, 17786–17791.

Lemke T, Welling A, Christel CJ, Blaich A, Bernhard D, Lenhardt P, Hofmann F & Moosmang S (2008). Unchanged $\beta$-adrenergic stimulation of cardiac L-type calcium channels in $Ca_V1.2$ phosphorylation site S1928A mutant mice. *J Biol Chem* **283**, 34738–34744.

Liman ER, Tytgat J & Hess P (1992). Subunit stoichiometry of a mammalian $K^+$ channel determined by construction of multimeric cDNAs. *Neuron* **9**, 861–871.

Link S, Meissner M, Held B, Beck A, Weissgerber P, Freichel M & Flockerzi V (2009). Diversity and developmental expression of L-type calcium channel $\beta$2 proteins and their influence on calcium current in murine heart. *J Biol Chem* **284**, 30129–30137.

Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G & Houslay MD (2005). RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with $\beta$-arrestin to control the protein kinase A/AKAP79-mediated switching of the $\beta$2-adrenergic receptor to activation of ERK in HEK293B2 cells. *J Biol Chem* **280**, 33178–33189.

McConnachie G, Langeberg LK & Scott JD (2006). AKAP signaling complexes: getting to the heart of the matter. *Trends Mol Med* **12**, 317–323.

Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK & Hescheler J (1999). Establishment of $\beta$-adrenergic modulation of L-type $Ca^{2+}$ current in the early stages of cardiomyocyte development. *Circ Res* **84**, 136–145.

Matsuda N, Hagiwara N, Shoda M, Kasanuki H & Hosoda S (1996). Enhancement of the L-type $Ca^{2+}$ current by mechanical stimulation in single rabbit cardiac myocytes. *Circ Res* **78**, 650–659.

Mikami A, Imoto K, Tanabe T, Niidome T, Mori Y, Takeshima H, Narumiya S & Numa S (1989). Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* **340**, 230–233.

Minobe E, Maeda S, Xu JJ, Hao LY, Kameyama A & Kameyama M (2014). A new phosphorylation site in cardiac L-type $Ca^{2+}$ channels (Cav1.2) responsible for its cAMP-mediated modulation. *Am J Physiol Cell Physiol* **307**, C999–C1009.

Miriyala J, Nguyen T, Yue DT & Colecraft HM (2008). Role of $Ca_V\beta$ subunits, and lack of functional reserve, in protein kinase A modulation of cardiac $Ca_V1.2$ channels. *Circ Res* **102**, e54–e64.

Morrow JP & Marx SO (2015). Novel approaches to examine the regulation of voltage-gated calcium channels in the heart. *Curr Mol Pharmacol* **8**, 61–68.

Nichols CB, Rossow CF, Navedo MF, Westenbroek RE, Catterall WA, Santana LF & McKnight GS (2010). Sympathetic stimulation of adult cardiomyocytes requires association of AKAP5 with a subpopulation of L-type calcium channels. *Circ Res* **107**, 747–756.

Norman RI & Leach RN (1994). Subunit structure and phosphorylation of the cardiac L-type calcium channel. *Biochem Soc Trans* **22**, 492–496.

Ohrtman JD, Romberg CF, Moua O, Bannister RA, Levinson SR & Beam KG (2015). Apparent lack of physical or functional interaction between $Ca_V1.1$ and its distal C terminus. *J Gen Physiol* **145**, 303–314.

Olsen SR & Uhler MD (1991). Isolation and characterization of cDNA clones for an inhibitor protein of cAMP-dependent protein kinase. *J Biol Chem* **266**, 11158–11162.

Osterrieder W, Brum G, Hescheler J, Trautwein W, Flockerzi V & Hofmann F (1982). Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates $Ca^{2+}$ current. *Nature* **298**, 576–578.

Oz S, Benmocha A, Sasson Y, Sachyani D, Almagor L, Lee A, Hirsch JA & Dascal N (2013). Competitive and non-competitive regulation of calcium-dependent inactivation in $Ca_V1.2$ L-type $Ca^{2+}$ channels by calmodulin and $Ca^{2+}$-binding protein 1. *J Biol Chem* **288** 12680–12691.

Pang L, Koren G, Wang Z & Nattel S (2003). Tissue-specific expression of two human $Ca_v1.2$ isoforms under the control of distinct 5′ flanking regulatory elements. *FEBS Lett* **546**, 349–354.

Pankonien I, Otto A, Dascal N, Morano I & Haase H (2012). Ahnak1 interaction is affected by phosphorylation of Ser-296 on $Ca_V\beta2$. *Biochem Biophys Res Commun* **421**, 184–189.

Parsons TD, Lagrutta A, White RE & Hartzell HC (1991). Regulation of $Ca^{2+}$ current in frog ventricular cardiomyocytes by 5′-guanylylimidodiphosphate and acetylcholine. *J Physiol* **432**, 593–620.

Patriarchi T, Qian H, Di Biase V, Malik ZA, Chowdhury D, Price JL, Hammes EA, Buonarati OR, Westenbroek RE, Catterall WA, Hofmann F, Xiang YK, Murphy GG, Chen CY, Navedo MF & Hell JW (2016). Phosphorylation of $Ca_v1.2$ on S1928 uncouples the L-type $Ca^{2+}$ channel from the $\beta_2$ adrenergic receptor. *EMBO J* **35**, 1330–1345.

Perez Reyes E, Yuan W, Wei X & Bers DM (1994). Regulation of the cloned L-type cardiac calcium channel by cyclic-AMP-dependent protein kinase. *FEBS Lett* **342**, 119–123.

Reuter H, Stevens CF, Tsien RW & Yellen G (1982). Properties of single calcium channels in cardiac cell culture. *Nature* **297**, 501–504.

Sako H, Green SA, Kranias EG & Yatani A (1997). Modulation of cardiac $Ca^{2+}$ channels by isoproterenol studied in transgenic mice with altered SR $Ca^{2+}$ content. *Am J Physiol* **273**, C1666–C1672.

Scott JD, Fischer EH, Takio K, Demaille JG & Krebs EG (1985). Amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase from rabbit skeletal muscle. *Proc Natl Acad Sci USA* **82**, 5732–5736.

Shannon KM, Klitzner TS, Chen F & Van Dop C (1995). Effects of triiodothyronine on retention of $\beta$-adrenergic responsiveness of voltage-gated transmembrane calcium current during culture of ventricular myocytes from neonatal rabbits. *Pediatr Res* **37**, 277–282.

Shistik E, Ivanina T, Blumenstein Y & Dascal N (1998). Crucial role of N terminus in function of cardiac L-type $Ca^{2+}$ channel and its modulation by protein kinase C. *J Biol Chem* **273**, 17901–17909.

Shistik E, Keren-Raifman T, Idelson GH, Dascal N & Ivanina T (1999). The N-terminus of the cardiac L-type $Ca^{2+}$ channel $\alpha_{1C}$ subunit: The initial segment is ubiquitous and crucial for protein kinase C modulation, but it is not directly phosphorylated. *J Biol Chem* **274**, 31145–31149.

Simms BA, Souza IA, Rehak R & Zamponi GW (2015). The $Ca_V1.2$ N terminus contains a CaM kinase site that modulates channel trafficking and function. *Pflugers Arch* **467**, 677–686.

Singer-Lahat D, Dascal N, Mittelman L, Peleg S & Lotan I (2000). Imaging plasma membrane proteins in large membrane patches of *Xenopus* oocytes. *Pflugers Arch* **440**, 627–633.

Singer-Lahat D, Lotan I, Biel M, Flockerzi V, Hofmann F & Dascal N (1994). Cardiac calcium channels expressed in Xenopus oocytes are modulated by dephosphorylation but not by cAMP-dependent phosphorylation. *Receptors Channels* **2**, 215–226.

Skeberdis VA, Jurevičius J & Fischmeister R (1997). $\beta2$ Adrenergic activation of L-type $Ca^{++}$ current in cardiac myocytes. *J Pharmacol Exp Ther* **283**, 452–461.

Snutch TP, Tomlinson WJ, Leonard JP & Gilbert MM (1991). Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. *Neuron* **7**, 45–57.

Tareilus E, Roux M, Qin N, Olcese R, Zhou J, Stefani E & Birnbaumer L (1997). A *Xenopus* oocyte $\beta$ subunit: evidence for a role in the assembly/expression of voltage-gated calcium channels that is separate from its role as a regulatory subunit. *Proc Natl Acad Sci USA* **94**, 1703–1708.

Taylor SS, Ilouz R, Zhang P & Kornev AP (2012). Assembly of allosteric macromolecular switches: lessons from PKA. *Nat Rev Mol Cell Biol* **13**, 646–658.

Thomsen MB, Foster E, Nguyen KH & Sosunov EA (2009). Transcriptional and electrophysiological consequences of KChIP2-mediated regulation of $Ca_V1.2$. *Channels (Austin)* **3**, 308–310.

Trautwein W, Taniguchi J & Noma A (1982). The effect of intracellular cyclic nucleotides and calcium on the action potential and acetylcholine response of isolated cardiac cells. *Pflugers Arch* **392**, 307–314.

Tröger J, Moutty MC, Skroblin P & Klussmann E (2012). A-kinase anchoring proteins as potential drug targets. *Br J Pharmacol* **166**, 420–433.

Uezono Y, Bradley J, Min C, McCarty NA, Quick M, Riordan JR, Chavkin C, Zinn K, Lester HA & Davidson N (1993). Receptors that couple to 2 classes of G proteins increase cAMP and activate CFTR expressed in Xenopus oocytes. *Receptors Channels* **1**, 233–241.

Viard P, Butcher AJ, Halet G, Davies A, Nurnberg B, Heblich F & Dolphin AC (2004). PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. *Nat Neurosci* **7**, 939–946.

Vinogradova TM & Lakatta EG (2009). Regulation of basal and reserve cardiac pacemaker function by interactions of cAMP-mediated PKA-dependent $Ca^{2+}$ cycling with surface membrane channels. *J Mol Cell Cardiol* **47**, 456–474.

Walsh DA, Ashby CD, Gonzalez C, Calkins D, Fischer EH & Krebs EG (1971). Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinases. *J Biol Chem* **246**, 1977–1985.

Wehrens XH, Lehnart SE & Marks AR (2005). Intracellular calcium release and cardiac disease. *Annu Rev Physiol* **67**, 69–98.

Wei X, Neely A, Lacerda AE, Olcese R, Stefani E, Perez Reyes E & Birnbaumer L (1994). Modification of $Ca^{2+}$ channel activity by deletions at the carboxyl terminus of the cardiac $\alpha_1$ subunit. *J Biol Chem* **269**, 1635–1640.

Weiss S, Keren-Raifman T, Oz S, Ben Mocha A, Haase H & Dascal N (2012). Modulation of distinct isoforms of L-type calcium channels by $G_q$-coupled receptors in *Xenopus* oocytes: Antagonistic effects of $G\beta\gamma$ and protein kinase C. *Channels* **6**, 426–437.

Weiss S, Oz S, Benmocha A & Dascal N (2013). Regulation of cardiac L-type $Ca^{2+}$ channel $Ca_V1.2$ via the $\beta$-adrenergic-cAMP-protein kinase A pathway: Old dogmas, advances, and new uncertainties. *Circ Res* **113**, 617–631.

Welsh MJ, Anderson MP, Rich DP, Berger HA, Denning GM, Ostedgaard LS, Sheppard DN, Cheng SH, Gregory RJ & Smith AE (1992). Cystic fibrosis transmembrane conductance regulator: A chloride channel with novel regulation. *Neuron* **8**, 821–829.

Wilkinson DJ, Strong TV, Mansoura MK, Wood DL, Smith SS, Collins FS & Dawson DC (1997). CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *Am J Physiol* **273**, L127–L133.

Yakubovich D, Berlin S, Kahanovitch U, Rubinstein M, Farhy Tselnicker I, Styr B, Keren-Raifman T, Dessauer CW & Dascal N (2015). A quantitative model of the GIRK1/2 channel reveals that its basal and evoked activities are controlled by unequal stoichiometry of $G\alpha$ and $G\beta\gamma$. *PLoS Comput Biol* **11**, e1004598.

Yang L, Dai D-F, Yuan C, Westenbroek RE, Yu H, West N, de la Iglesia HO & Catterall WA (2016). Loss of $\beta$-adrenergic-stimulated phosphorylation of $Ca_V1.2$ channels on Ser1700 leads to heart failure. *Proc Natl Acad Sci USA* **113**, E7976–E7985.

Yang L, Katchman A, Samad T, Morrow JP, Weinberg RL & Marx SO (2013). $\beta$-Adrenergic regulation of the L-type $Ca^{2+}$ channel does not require phosphorylation of $\alpha_{1C}$ Ser1700. *Circ Res* **113**, 871–880.

Yue DT, Herzig S & Marban E (1990). $\beta$-Adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. *Proc Natl Acad Sci USA* **87**, 753–757.

Zong X, Schreieck J, Mehrke G, Welling A, Schuster A, Bosse E, Flockerzi V & Hofmann F (1995). On the regulation of the expressed L-type calcium channel by cAMP-dependent phosphorylation. *Pflugers Arch* **430**, 340–347.

## Additional information

### Competing interests

None declared.

### Author contributions

The experiments were performed in the Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University; Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany; and Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, Homburg, Germany. S.O., I.P., H.H., V.F., E.K. and N.D. participated in the conception and design of the study, interpretation of the results and in editing the draft of the paper. A.B., I.P. and S.O. prepared the DNA constructs. S.O. and I.P. performed the experiments and analysed the data. S.O. and N.D. wrote the paper. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors qualify for authorship, and all those who qualify for authorship are listed.

### Funding

This study was supported by the German-Israel Foundation for Research and Development (GIF; N.D., H.H. and E.K., grant no. I-1210-286.13/2012), the Fields Fund for Molecular Cardiology (N.D.), and Sonderforschungsbereich (SFB) 894 by the German Research Foundation (V.F.).

### Acknowledgements

We thank Dr Ronit S. Cherki for performing some of the two-electrode voltage clamp experiments, Dr Sharon Weiss for critical reading of the manuscript, and the colleagues who kindly provided the cDNA constructs: W. A. Catterall (AKAP15), H. A. Lester (CFTR) and M. D. Uhler (PKI). This work was performed in partial fulfilment of the requirements for the PhD degree of S. Oz.

### Authors' present addresses

S. Oz: Leviev Heart Center, Sheba Medical Center, Tel Hashomer, Ramat Gan, Israel.

I. Pankonien: University of Lisbon, Faculty of Sciences, BioISI–Biosystems and Integrative Sciences Institute, Campo Grande, 1749-016, Lisbon, Portugal.