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## Ca<sup>2+</sup>/Calcineurin-Dependent Inactivation of Neuronal L-type Ca<sup>2+</sup> Channels Requires Priming by AKAP-Anchored Protein Kinase A

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### SUMMARY

Within neurons, Ca<sup>2+</sup>-dependent inactivation (CDI) of voltage-gated L-type Ca<sup>2+</sup> channels shapes cytoplasmic Ca<sup>2+</sup> signals. CDI is initiated by Ca<sup>2+</sup> binding to channel-associated calmodulin and subsequent Ca<sup>2+</sup>/calmodulin activation of the Ca<sup>2+</sup>-dependent phosphatase, calcineurin (CaN), which is targeted to L channels by the A-kinase anchoring protein, AKAP79/150. Here we report that CDI of neuronal L channels was abolished by inhibition of PKA activity or PKA anchoring to AKAP79/150, and that CDI was also suppressed by stimulation of PKA activity. Although CDI was reduced by positive or negative manipulation of PKA, interference with PKA anchoring or activity lowered Ca<sup>2+</sup> current density whereas stimulation of PKA activity elevated it. In contrast, inhibition of CaN reduced CDI but had no effect on current density. These results suggest a model wherein PKA-dependent phosphorylation enhances neuronal L current, thereby priming channels to undergo CDI, and Ca<sup>2+</sup>/calmodulin-activated CaN actuates CDI by reversing PKA-mediated enhancement of channel activity.

### INTRODUCTION

Voltage-gated Ca<sup>2+</sup> channels convert patterns of electrical activity on the neuronal surface membrane into signals that can initiate intracellular signaling: rises in cytoplasmic Ca<sup>2+</sup>. Within neurons, Ca<sup>2+</sup> can trigger release of neurotransmitter and changes in gene expression that contribute to modification of cell morphology and synaptic plasticity (Catterall, 2011). Residing at the interface between electrical and chemical signaling, Ca<sup>2+</sup> channels represent natural points for regulation, with up-modulation and down-modulation of channel activity providing precise spatiotemporal control of cytoplasmic Ca<sup>2+</sup> signals that specify which of

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### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, which can be found with this article online.

various  $\text{Ca}^{2+}$ -dependent processes are activated, and how strongly. Curbing  $\text{Ca}^{2+}$  channel activity is also critical in avoiding cytotoxicity arising from  $\text{Ca}^{2+}$  overload (Choi, 1994; Nägerl et al., 2000). One important mechanism that has evolved to limit  $\text{Ca}^{2+}$  entry via  $\text{Ca}^{2+}$  channels is  $\text{Ca}^{2+}$ -dependent inactivation (CDI; Tillotson, 1979; Budde et al., 2002).

Calmodulin (CaM) has been identified as the  $\text{Ca}^{2+}$  sensor that initiates CDI (Zühlke et al., 1999; Peterson et al., 1999), and in the CaM-actuated model of CDI,  $\text{Ca}^{2+}$  ions entering the cytoplasm bind to calmodulin docked on the channel through which they have just passed;  $\text{Ca}^{2+}$ /CaM undergoes a conformational change that is sensed by its associated channel; and the channel is nudged into an inactivated conformation incapable of conducting  $\text{Ca}^{2+}$  (Erickson et al., 2003). Despite the elegance of studies aimed at elucidating the mechanism of CaM-actuated CDI, they generally have had the major drawback of relying upon heterologous expression of voltage-gated  $\text{Ca}^{2+}$  channels in cells that naturally lack these channels and are also deficient in the scaffolding proteins that target enzymes like PKA and CaN to channels. Using a more intact and physiologically relevant system of cultured hippocampal neurons, we recently described experimental results strongly suggesting that  $\text{Ca}^{2+}$ /CaM initiates CDI largely through activation of the natural  $\text{Ca}^{2+}$ /CaM substrate, CaN (Oliveria et al., 2012). We found that CaN, anchored to  $\text{Ca}_v1.2$  by the A-kinase anchoring protein AKAP79/150 (human/rodent), was essential for CDI of pharmacologically-isolated L-type  $\text{Ca}^{2+}$  current in hippocampal neurons.

Disruption of this anchoring protein prevented enhancement by PKA of L-current amplitude in cultured neurons, raising the possibility that PKA might enhance L-current by opposing CaM/CaN-mediated CDI. Modulation of  $\text{Ca}_v1.2$  by PKA is one of the best-described forms of ion channel modulation, and has been identified in a variety of excitable cell types (Bean et al., 1984; Kalman et al., 1988; Hadley and Lederer, 1991; Rankovic et al., 2011).

Here, we report evidence from hippocampal neurons indicating that impairment of PKA anchoring or activity decreases L-type  $\text{Ca}^{2+}$  current density and abolishes CDI of these channels. Furthermore, neurons in which PKA activity was stimulated exhibited concomitant enhancement of current and diminution of CDI. These experimental results can be explained by a simple model of inverse control by PKA and CaN of L channel current and kinetics: PKA-dependent phosphorylation enhances L channel opening probability and primes channels for CDI, and  $\text{Ca}^{2+}$ /CaM-activated CaN actuates CDI by reversing PKA-mediated enhancement. This mechanism readily accommodates the experimental observations that interference with the action of either PKA or CaN obstructs the normal process of CDI. More generally, these results expand the repertoire of L-channel-complexed proteins known to modulate  $\text{Ca}^{2+}$  signals in postsynaptic regions: channel-bound CaM and AKAP79/150-anchored CaN and PKA function coordinately to tune  $\text{Ca}^{2+}$  signals that regulate neuronal gene expression, as further explored in a companion paper (Murphy et al. submitted to Cell Reports).

## RESULTS

### Channel-localized PKA enhances current density and primes channels for CDI

In rodent hippocampal pyramidal neurons grown in culture for up to 5 days,  $\text{Ca}^{2+}$  current carried by L-type channels exhibited two components of inactivation: fast,  $\text{Ca}^{2+}$ -dependent inactivation ( $1/\tau = 40.6 \pm 2.1 \text{ s}^{-1}$  in mice, Fig. 1A, *red bars*;  $42.9 \pm 2.0 \text{ s}^{-1}$  in rats (Oliveria et al., 2012)) and slow, voltage-dependent inactivation that remains present when  $\text{Ba}^{2+}$  (*black bars*) is substituted for  $\text{Ca}^{2+}$  in the extracellular solution. The fast component—CDI—was virtually eliminated in AKAP150-knockout mice (AKAP150<sup>-/-</sup>; Fig. 1A), consistent with previously-reported results with RNAi-mediated knock-down of AKAP150 (150RNAi) in rat neurons (Oliveria et al., 2007; Oliveria et al., 2012). CDI was fully restored in 150RNAi knock-down neurons that were co-transfected with the 150RNAi-insensitive human ortholog of AKAP150, AKAP79 (79wt + 150RNAi), confirming the importance of AKAP79/150 in L-channel CDI.

Knock-down or knockout of AKAP150 de-localizes both CaN and PKA from the L-channel nano-environment, which precludes identification of CaN- or PKA-specific effects on channel function. To probe more specifically whether AKAP-anchored PKA is involved in CDI, L currents were recorded from neurons that express an AKAP79/150 mutant incapable of anchoring PKA, owing to a selective deletion within the PKA-RII subunit binding domain (residues 391-400; designated 79 PKA) (Carr et al., 1992; Oliveria et al., 2003; Murphy et al., submitted to Cell Reports). Rat neurons co-transfected with AKAP79 PKA (79 PKA) and 150RNAi to knock down native AKAP150 exhibited greatly slowed CDI (Fig. 1A, row 2). Neurons cultured from AKAP150 PKA knockin mice (Murphy et al. submitted to Cell Reports) showed similarly-slowed CDI (Fig. 1A, row 5). CDI was also significantly slowed in neurons expressing 150RNAi and 79pro2 (Fig. 1A, row3), an AKAP79 mutant bearing two proline substitutions (A<sub>396</sub>P and I<sub>404</sub>P) that disrupt the helical structure of the PKA binding site (Carr et al., 1992; Oliveria et al., 2007).

The fraction of peak current remaining 400 ms after the onset of step depolarization ( $r_{400}$ ) provides another view of inactivation, and the difference between  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  in fractional current remaining 400 ms after the onset of depolarization ( $r_{400,\text{Ba}} - r_{400,\text{Ca}}$ ) provides a second useful index of CDI (Fig. 1B, top) (Kim et al., 2004; Oz et al., 2011). A third CDI metric estimates the fraction of  $\text{Ca}^{2+}$  prevented from entering the neuron as a consequence of CDI: peak  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  current amplitudes were normalized because  $\text{Ba}^{2+}$  permeates the channel more readily, then the peak-normalized  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  currents were integrated to obtain relative charge transfer, and the difference between these ( $\hat{A}_{\text{Ba}} - \hat{A}_{\text{Ca}}$ ) approximates the fractional depression of  $\text{Ca}^{2+}$  entry by CDI (Fig. 1B, bottom). For the AKAP manipulations studied, the patterns for both CDI index and depression of  $\text{Ca}^{2+}$  entry ( $\hat{A}_{\text{Ba}} - \hat{A}_{\text{Ca}}$ ) mirrored that for rate of CDI ( $1/\tau$ ) in Fig. 1A.

Deficits in AKAP79/150 anchoring of PKA (79 PKA, pro2) that reduced CDI also reduced peak  $\text{Ca}^{2+}$  current density (Fig. 1C, top). In contrast, current density was not decreased in cells expressing mutant AKAP79/150 in which the CaN-binding PxIxIT-like motif (PIX mutants) was deleted. The overall pattern for integrated  $\text{Ca}^{2+}$  current density, i.e.  $\text{Ca}^{2+}$  charge density (Fig. 1C, bottom), is similar to the peak current density pattern, but less

pronounced: disruption of PKA anchoring moderately reduced charge density, whereas disruption of CaN anchoring increased L-channel charge density, albeit modestly. Peak current density and charge density provide different views of channel activity, but together, they suggest the idea that anchored PKA supports a basal degree of enhancement of L-channel activity that is limited by anchored CaN. Using  $\text{Ca}^{2+}$  imaging and phospho-immunoblotting, our companion paper in this issue (Murphy et al., submitted to Cell Reports) provides additional evidence that disruption of AKAP79/150-PKA anchoring leads to substantial decreases in basal L-channel phosphorylation and  $\text{Ca}^{2+}$  signaling in hippocampal neurons.

Inward  $\text{Ca}^{2+}$  current increases with increasing depolarization, and then declines to zero as membrane potential approaches the reversal potential for L channels; as a  $\text{Ca}^{2+}$ -dependent process, the degree of CDI reflects the trajectory of this  $I_{\text{Ca}}-V_{\text{M}}$  curve (Fig. 1D). L currents measured from 150RNAi/79 PKA neurons were smaller and inactivated more slowly than in 150RNAi/79wt control neurons, but the voltage-dependence of both current density and  $1/\tau$  for CDI showed the same form as for control, indicating that the decreased current density caused by disruption of PKA anchoring cannot be attributed to a shift in the channel's voltage-dependence of gating. Further, the large reduction in CDI caused by disruption of PKA anchoring cannot be explained by the more modest reduction in current density that was caused by this anchoring disruption.

If our explanation of the effects of PKA on CDI was correct, we predicted that pharmacological antagonism of either PKA anchoring to the AKAP or of PKA enzymatic activity would diminish  $\text{Ca}^{2+}$  current density and CDI. To test this prediction,  $\text{Ca}^{2+}$  currents were measured when PKA anchoring was antagonized by Ht31, an AKAP-derived peptide that binds the RII regulatory subunit of PKA with nanomolar affinity and thereby competes with AKAPs for binding to RII (Carr et al., 1992). By incorporating Ht31 into the whole-cell pipet, we found that Ht31 dose-dependently reduced peak current density and CDI, with an  $IC_{50}$  of 5-10  $\mu\text{M}$  (Fig. 2A, B). Current density and CDI were insensitive to a high concentration of a proline substitution mutant of Ht31 (Ht31-Pro) that cannot properly bind to PKA RII (Fig. 2A). In another set of experiments, PKA activity was inhibited by loading the PKA inhibitory peptide, PKI, into the whole-cell recording pipets. Inhibition of PKA activity also depressed  $\text{Ca}^{2+}$  current density and eliminated CDI (Fig. 2C). Thus competitive inhibitors of PKA anchoring and activity acted much like the genetic manipulations that impaired PKA anchoring, and as predicted, the competitive inhibitors reduced current density and attenuated CDI.

### PKA versus CaN in CDI

To examine the connection between PKA and CaN in CDI of neuronal L channels, we tested the effects on whole-cell  $\text{Ca}^{2+}$  currents of fast application of membrane-permeant inhibitors of PKA (H89) or CaN (FK506). After establishing a baseline for peak inward  $\text{Ca}^{2+}$  current density, the solution flowing over the neuron was quickly changed from control to 500 nM H89 ( $K_i = 135$  nM; Davies et al., 2000). H89 application lowered both peak current density and CDI rate to new steady levels in control neurons (Fig. 3A, left), but not in 79 PKA neurons (Fig. 3A, right). The low density of current in 150RNAi/79 PKA neurons, near

absence of CDI, and lack of effect of H89 are consistent with results in Figs. 1 and 2: in 79 PKA neurons, channels are already largely de-enhanced and inactivated. Antagonism of CaN via fast application of 5  $\mu$ M FK506 to control neurons slowed CDI but had no significant effect on peak current density (Fig. 3B, left). In 150RNAi/79 PIX neurons, which have disrupted CaN anchoring, FK506 exerted no obvious effect on current density, or rate of CDI (Fig. 3B, right). But in this case, current density remained high, while CDI was low, as though channels were in an enhanced state but no longer subject to CDI. In both situations, the experimental results with drugs that altered PKA or CaN activity agreed with results obtained with AKAP mutants bearing binding site deletions for PKA or CaN.

### Elevation of PKA activity suppresses CDI

Compared to non-stimulated control neurons, forskolin-stimulated neurons with concomitant elevated PKA activity exhibited significantly less CDI and enhanced L current density (Fig. 4A). If attenuation of CDI reflected the ability of an elevated level of PKA activity to overcome CaN action, then other means of stimulating PKA were predicted to reduce CDI as well. We therefore tested the effects on L current of fast application of a potent and membrane-permeant cAMP analogue, Sp-5,6-dichloro-cBIMPS (Sandberg et al., 1991). At 100  $\mu$ M, but not 10  $\mu$ M, the cAMP analogue significantly increased peak L current density (~23%) and reduced CDI, as predicted (Fig. 4B).

## DISCUSSION

For endogenous L-channel complexes in neurons, we have found that  $Ca^{2+}$ /CaN-mediated CDI requires priming of L channels by PKA. A PKA-dependence for CDI was revealed by loss of CDI when either PKA activity was pharmacologically inhibited or PKA anchoring to AKAP79/150 was prevented. Three distinct experimental approaches to disrupt PKA anchoring—(i) 150 PKA knock-in, (ii) 150RNAi knockdown and replacement by PKA-binding-defective AKAP79 PKA or AKAP79pro2, or (iii) competitive inhibition of PKA anchoring by Ht31—abolished CDI, and thus we conclude that PKA anchoring by AKAP79/150 is a prerequisite for CDI (Fig. 1; Fig. 2A-B). Whether determined from rate of CDI, CDI index, or fractional depression of  $Ca^{2+}$  entry, all anchoring interventions strongly reduced CDI in cultured neurons.

Kinase activity of PKA is also a prerequisite for CDI of L channels in neurons: CDI was prevented either by a synthetic mimic of a highly-selective natural inhibitor of free PKA catalytic subunits, PKI-(6-22)-amide (Fig. 2C), or by a low concentration of a competitive inhibitor of ATP binding in the enzyme's catalytic site, 500 nM H89 (Fig. 3A) (Murray, 2008). H89 was without effect in knockdown/rescue experiments in which PKA anchoring was disrupted, indicating that H89 suppression of CDI is attributable specifically to antagonism of PKA that is anchored in the channel-AKAP complex.

CDI was efficaciously suppressed by the calcineurin inhibitor FK506 (Fig. 3B), directly in line with the idea that CDI of neuronal L channels is carried out by calcineurin (Chad and Eckert, 1986; Armstrong and Eckert, 1987; Oliveria et al., 2007; Oliveria et al., 2012). In calcineurin anchoring-defective 79 PIX neurons, the absence of calcineurin from L channel complexes resulted in large L channel  $Ca^{2+}$  currents with little CDI. FK506 had no effect on

the (already-diminished) CDI of L channels in 79 PIX neurons, presumably because calcineurin, the target of FK506 action, was not present in the AKAP/L-channel complex.

### Channel-localized PKA activity supports CaN-mediated CDI

Experimental manipulations that impaired either anchoring (PKA or Ht31) or activity (PKI, H89) of PKA virtually abolished CDI, but they also decreased current density (from 18-20 pA/pF to 10-14 pA/pF). Was loss of CDI in these cases simply a consequence of smaller  $Ca^{2+}$  influx rather than decreased phosphorylation by PKA? Apparently not, because strong CDI was evident in WT currents recorded at  $-10$  mV or  $+30$  mV, and at these potentials WT currents were similar in amplitude to those of CDI-lacking 79 PKA currents recorded at the peak ( $+10$  mV) of the current-voltage relationship (Fig. 1D). Therefore, loss of CDI upon disruption of PKA anchoring or block of PKA activity apparently arose as a direct consequence of the loss of channel-localized PKA activity.

PKI and disruption of PKA localization were very similar to one another in the size of their effects on current density ( $\sim 30$ - $40\%$  depression) and CDI (abolishment). This similarity indicates that an intact pool of PKA anchored by AKAP79/150 to L channels has a basal level of activity in neurons, that this pool of PKA enhances L current, and that this degree of PKA-dependent enhancement is sufficient to support subsequent CaN-mediated CDI. For Ht31 treatment, de-enhancement of current and suppression of CDI displayed overlapping concentration-response relationships, suggesting that these two effects are directly related to one another (Fig. 2B). Regulation of L channels by basal activity of AKAP79/150-anchored PKA may be attributable to cAMP-independent kinase activity of catalytic subunits in type II PKA holoenzymes or to activity of catalytic subunits that may remain associated with RII subunits in cAMP-activated PKA holoenzymes (Yang et al., 1995; Smith et al., 2013).

### Phosphorylation is a prerequisite for CaN-mediated, dephosphorylation-dependent CDI

We have found that stimulation of PKA activity (FSK, cBIMPS) in hippocampal neurons reduced CDI (Fig. 4). Similarly, other groups have found that the beta agonist isoproterenol, forskolin, mimics of cAMP or intracellular perfusion with PKA reduced CDI of voltage-gated  $Ca^{2+}$  channels in neurons (Kalman et al., 1988; You et al., 1995). Yet we have found that interference with PKA action (de-localization, block) also reduced CDI, much like stimulation of PKA did. Thus CDI was reduced by either positive or negative manipulation of PKA action. In contrast, the effects of PKA manipulation on current density were straightforward: interference with PKA localization or activity reduced current density whereas stimulation of PKA (by forskolin or cBIMPS) increased current density. How might the effects of PKA manipulation upon CDI and enhancement of L current be reconciled, and in particular, how might interference with localized PKA action reduce CDI?

Evidently, PKA-mediated enhancement and CaN-mediated CDI function as inverse operations at L channels: PKA enhances via phosphorylation, and CaN de-enhances – initiates CDI – via dephosphorylation. A natural conclusion is that PKA-dependent phosphorylation primes channels to undergo CDI, and  $Ca^{2+}$ /CaM-activated CaN actuates CDI by reversing PKA-mediated enhancement. In this model, elevated PKA activity (FSK, cBIMPS) overcomes CaN phosphatase action and thereby reduces CDI and enhances

current. Conversely, when channel-localized PKA action is impeded ( PKA, Pro2, Ht31, H89, PKI), the diminution of current density and of CDI reflect the facts that channel opening probability is reduced and fewer channels are phosphorylated and thus available to undergo CaN-dependent CDI. In essence, impediment of PKA action allows the CDI process to chronically dominate channel gating, an increased fraction of channels resides in a Ca<sup>2+</sup>-inactivated state, and fewer open channels remain available to undergo CDI. More complex alternative explanations are possible as well. The time course of the effects of H89 on L current particularly supports our simple interpretation (Fig. 3A). The time course of FK506 action can also be accommodated by this model: during FK506 inhibition of Ca<sup>2+</sup>/CaM-activated CaN, L-channel current density was maintained and presumably the number of phosphorylated, active L channels was preserved as well; at the same time, the Ca<sup>2+</sup>/CaN-mediated dephosphorylation of channels that subserves CDI was prevented, thus reducing rate and degree of CDI.

In summary, our work addresses the control of postsynaptic L-type Ca<sup>2+</sup> signals, and particularly the mechanism of CDI in cultured hippocampal neurons. In these neurons, CDI requires prior priming of channels by PKA, with the actual process of CDI mediated by CaM and CaN (Chad and Eckert, 1986; Armstrong, 1989; Oliveria et al., 2007; Oliveria et al., 2012). In the PKA-CaN network that regulates L channel activity, PKA may be activated by an upstream input, for example by  $\beta_2$  adrenergic receptors (Gray and Johnston, 1987; Davare et al., 2001; Hoogland and Saggau, 2004), which provides a positive modulatory influence on L channels. PKA might also be activated by Ca<sup>2+</sup>/CaM-stimulated adenylyl cyclase activity, which would provide positive autoregulatory feedback onto L channels. Negative autoregulatory feedback is provided by Ca<sup>2+</sup>/CaM-activated CaN, in the form of CDI. Such integrated control by PKA and CaN of L-channel Ca<sup>2+</sup> signals is also critical in regulation of gene expression in neurons. For example, a companion paper in this issue (Murphy et al., submitted to Cell Reports) demonstrates that anchoring of PKA within the AKAP/L channel complex is necessary to balance the inhibitory action on L channels of co-anchored-CaN, and thereby sustain proper neuronal excitation-transcription coupling.

## EXPERIMENTAL PROCEDURES

Hippocampal pyramidal neurons obtained from neonatal rats and mice were cultured for up to 5 days, in order to reduce the more egregious loss of space-clamp that occurs in longer-term cultured neurons exhibiting extensive neuritic branching. Compared to older cultures, the short-term cultured neurons exhibited faster activation and deactivation of L-type currents, facilitating measurement of inactivation kinetics. Details regarding preparation of short-term cultured hippocampal pyramidal neurons, patch-clamp recording and data analysis are described in the Supplemental Experimental Procedures.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## REFERENCES

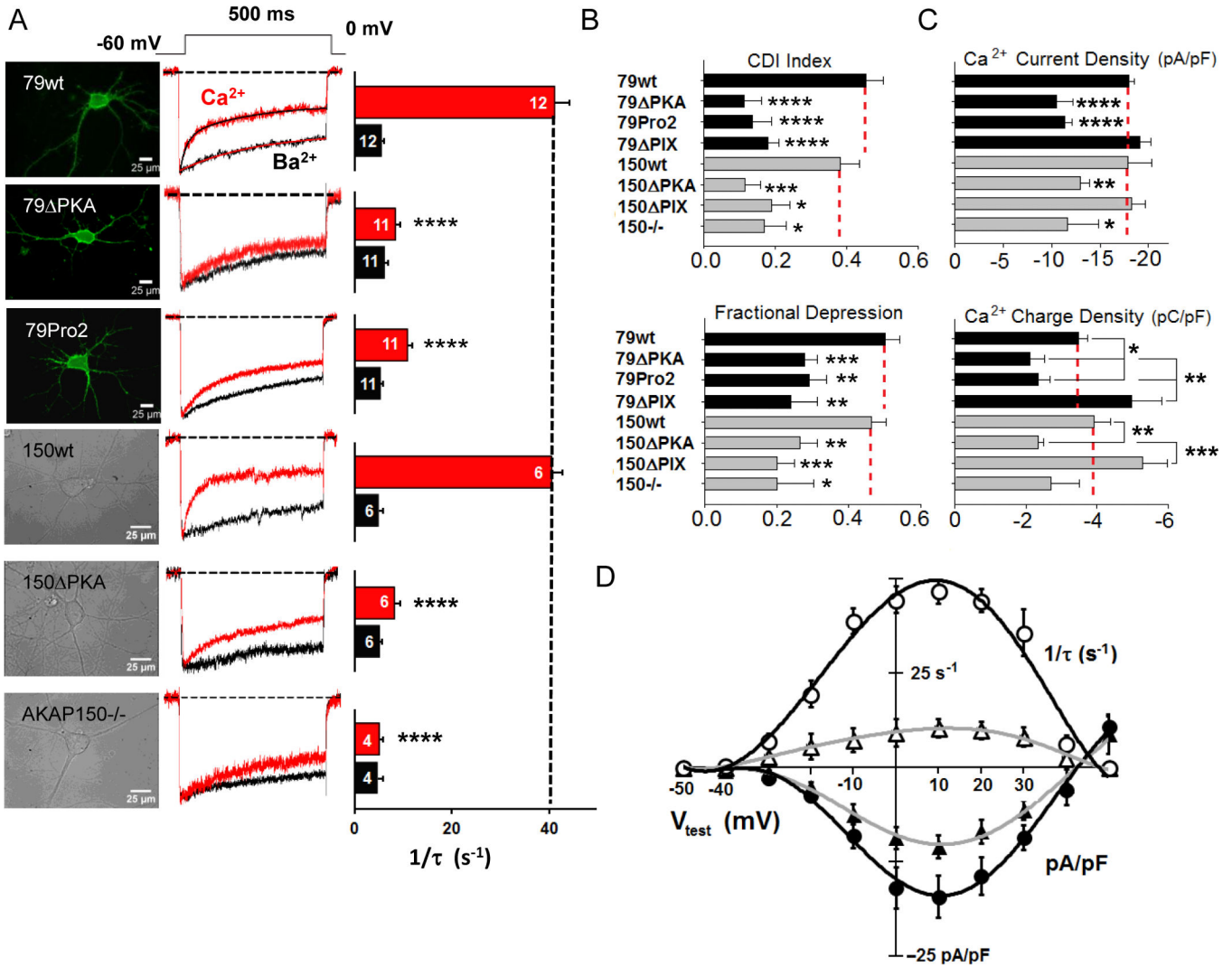
- Armstrong D, Eckert R. Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA*. 1987; 84:2518–2522. [PubMed: 2436233]
- Armstrong DL. Calcium channel regulation by calcineurin, a Ca<sup>2+</sup>-activated phosphatase in mammalian brain. *Trends Neurosci*. 1989; 12:117–122. [PubMed: 2469218]
- Augustine GJ, Santamaria F, Tanaka K. Local calcium signaling in neurons. *Neuron*. 2003; 40:331–346. [PubMed: 14556712]
- Bauman AL, Goehring AS, Scott JD. Orchestration of synaptic plasticity through AKAP signaling complexes. *Neuropharmacology*. 2004; 46:299–310. [PubMed: 14975685]
- Bean BP, Nowycky MC, Tsien RW.  $\beta$ -Adrenergic modulation of calcium channels in frog ventricular heart cells. *Nature*. 1984; 307:371–375. [PubMed: 6320002]
- Budde T, Meuth S, Pape HC. Calcium-dependent inactivation of neuronal calcium channels. *Nat. Rev. Neurosci*. 2002; 3:873–883. [PubMed: 12415295]
- Carr DW, Hausken ZE, Fraser ID, Stofko-Hahn RE, Scott JD. 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*. 1992; 267:13376–13382. [PubMed: 1618839]
- Catterall WA. Voltage-gated calcium channels. *Cold Spring Harb. Perspect. Biol*. 2011; 3:a003947. [PubMed: 21746798]
- Chad JE, Eckert R. An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. *J. Physiol*. 1986; 378:31–51. [PubMed: 2432251]
- Choi DW. Calcium and excitotoxic neuronal injury. *Ann. N. Y. Acad. Sci*. 1994; 747:162–171. [PubMed: 7847669]
- Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW. A  $\beta_2$  adrenergic receptor signaling complex assembled with the Ca<sup>2+</sup> channel Cav1.2. *Science*. 2001; 293:98–101. [PubMed: 11441182]
- Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J*. 2000; 351:95–105. [PubMed: 10998351]
- Erickson MG, Liang H, Mori MX, Yue DT. FRET Two-hybrid mapping reveals function and location of L-Type Ca<sup>2+</sup> channel CaM preassociation. *Neuron*. 2003; 39:97–107. [PubMed: 12848935]
- Gray R, Johnston D. Noradrenaline and  $\beta$ -adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature*. 1987; 327:620–622. [PubMed: 2439913]
- Hadley RW, Lederer WJ. Ca<sup>2+</sup> and voltage inactivate Ca<sup>2+</sup> channels in guinea-pig ventricular myocytes through independent mechanisms. *J. Physiol*. 1991; 444:257–268. [PubMed: 1668348]
- Hoogland TM, Saggau P. Facilitation of L-type Ca<sup>2+</sup> channels in dendritic spines by activation of  $\beta_2$  adrenergic receptors. *J. Neurosci*. 2004; 24:8416–8427. [PubMed: 15456814]
- Kalman D, O'Laugh PH, Erxleben C, Armstrong DL. Calcium-dependent inactivation of the dihydropyridine-sensitive calcium channels in GH3 cells. *J. Gen. Physiol*. 1988; 92:531–548. [PubMed: 2849631]
- Kim J, Ghosh S, Nunziato DA, Pitt GS. Identification of the components controlling inactivation of voltage-gated Ca<sup>2+</sup> channels. *Neuron*. 2004; 41:745–754. [PubMed: 15003174]
- Lee A, Scheuer T, Catterall WA. Ca<sup>2+</sup>/calmodulin-dependent facilitation and inactivation of P/Q-type Ca<sup>2+</sup> channels. *J. Neurosci*. 2000; 20:6830–6838. [PubMed: 10995827]
- Murphy, JG.; Sanderson, JL.; Gorski, JA.; Scott, JD.; Catterall, WA.; Sather, WA.; Dell'Acqua, ML. AKAP-anchored PKA maintains neuronal L-type calcium channel activity and NFAT transcriptional signaling. 2013. (submitted to Cell Reports)



- Murray AJ. Pharmacological PKA inhibition: all may not be what it seems. *Sci. Signal.* 2008; 1:re4. [PubMed: 18523239]
- Nägerl UV, Mody I, Jeub M, Lie AA, Elger CE, Beck H. Surviving granule cells of the sclerotic human hippocampus have reduced  $\text{Ca}^{2+}$  influx because of a loss of calbindin-D(28k) in temporal lobe epilepsy. *J. Neurosci.* 2000; 20:1831–1836. [PubMed: 10684884]
- Oliveria SF, Gomez LL, Dell'Acqua ML. Imaging kinase--AKAP79--phosphatase scaffold complexes at the plasma membrane in living cells using FRET microscopy. *J. Cell. Biol.* 2003; 160:101–112. [PubMed: 12507994]
- Oliveria SF, Dell'Acqua ML, Sather WA. AKAP79/150 anchoring of calcineurin controls neuronal L-type  $\text{Ca}^{2+}$  channel activity and nuclear signaling. *Neuron.* 2007; 55:261–275. [PubMed: 17640527]
- Oliveria SF, Dittmer PJ, Youn DH, Dell'Acqua ML, Sather WA. Localized calcineurin confers  $\text{Ca}^{2+}$ -dependent inactivation on neuronal L-type  $\text{Ca}^{2+}$  channels. *J. Neurosci.* 2012; 32:15328–15337. [PubMed: 23115171]
- Oz S, Tsemakhovich V, Christel CJ, Lee A, Dascal N. CaBP1 regulates voltage-dependent inactivation and activation of  $\text{Ca}_v1.2$  (L-type) calcium channels. *J. Biol. Chem.* 2011; 286:13945–13953. [PubMed: 21383011]
- Peterson BZ, DeMaria CD, Adelman JP, Yue DT. Calmodulin is the  $\text{Ca}^{2+}$  sensor for  $\text{Ca}^{2+}$ -dependent inactivation of L-type calcium channels. *Neuron.* 1999; 22:549–558. [PubMed: 10197534]
- Pitt GS. Calmodulin and CaMKII as molecular switches for cardiac ion channels. *Cardiovasc. Res.* 2007; 73:641–647. [PubMed: 17137569]
- Rankovic V, Landgraf P, Kanyshkova T, Ehling P, Meuth SG, Kreutz MR, Budde T, Munsch T. Modulation of calcium-dependent inactivation of L-type  $\text{Ca}^{2+}$  channels via beta-adrenergic signaling in thalamocortical relay neurons. *PLoS One.* 2011; 6:e27474. [PubMed: 22164209]
- Sandberg M, Butt E, Nolte C, Fischer L, Halbrugge M, Beltman J, Jahnsen T, Genieser HG, Jastorff B, Walter U. Characterization of Sp-5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (Sp-5,6-DC1-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem. J.* 1991; 279:521–527. [PubMed: 1659381]
- Smith FD, Reichow SL, Esseltine JL, Shi D, Langeberg LK, Scott JD, Gonen T. Intrinsic disorder within an AKAP-protein kinase A complex guides local substrate phosphorylation. *eLife.* 2013; 2:e01319. [PubMed: 24192038]
- Tillotson D. Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. Natl. Acad. Sci. USA.* 1979; 76:1497–1500. [PubMed: 286336]
- Yang S, Fletcher WH, Johnson DA. Regulation of cAMP-dependent protein kinase: enzyme activation without dissociation. *Biochemistry.* 1995; 34:6267–6271. [PubMed: 7756252]
- You Y, Pelzer DJ, Pelzer S. Trypsin and forskolin decrease the sensitivity of L-type calcium current to inhibition by cytoplasmic free calcium in guinea pig heart muscle cells. *Biophys. J.* 1995; 69:1838–1846. [PubMed: 8580327]
- Zühlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H. Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature.* 1999; 399:159–162. [PubMed: 10335846]

**HIGHLIGHTS**

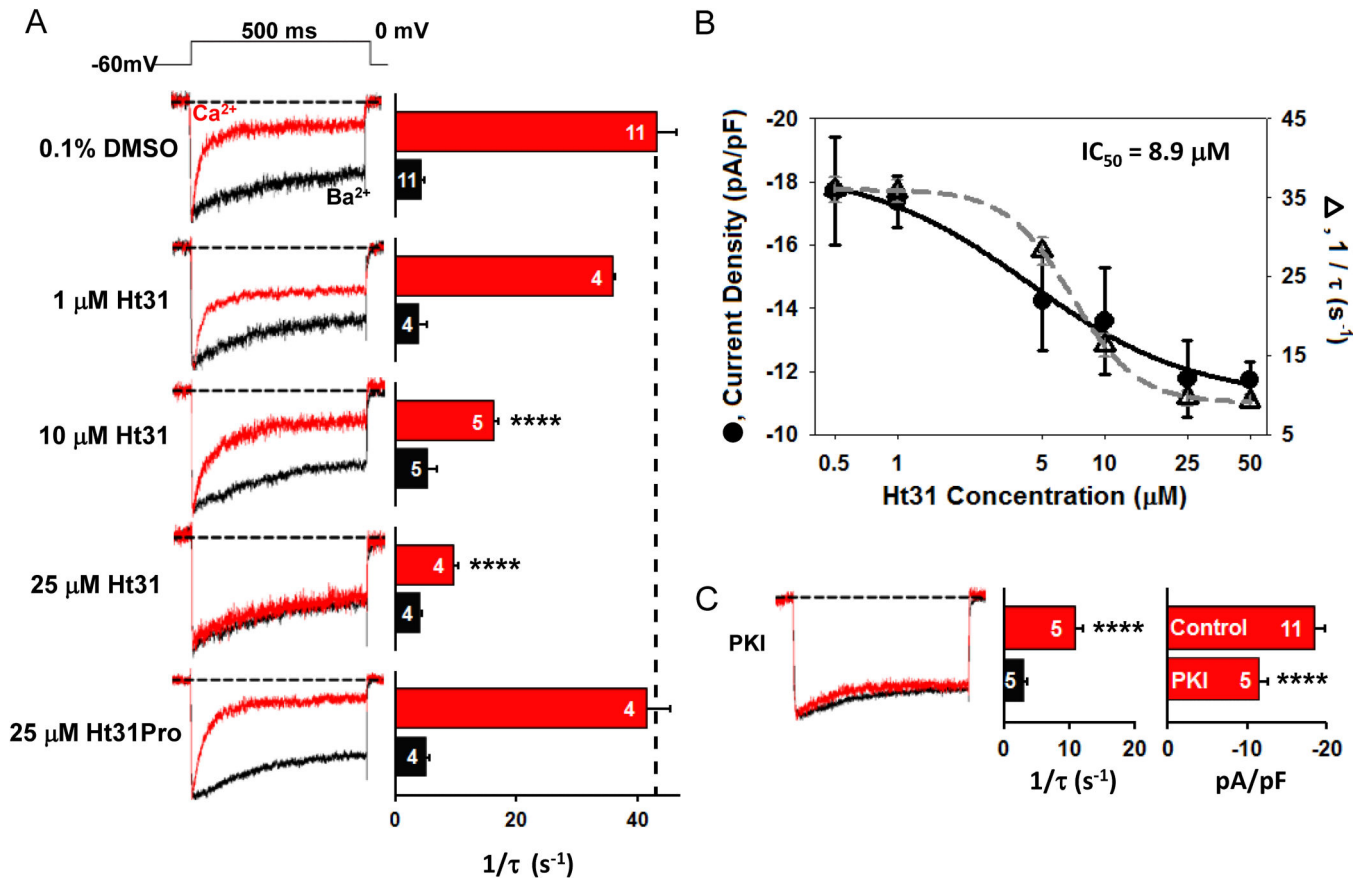
- $\text{Ca}^{2+}$ /CaN-mediated CDI of neuronal L channels requires a basal level of PKA activity
- CDI of neuronal L channels relies upon AKAP79/150-anchored PKA
- CDI is attenuated by either elevation or obstruction of PKA action on L channels
- L channels must be primed by PKA to undergo  $\text{Ca}^{2+}$ /calcineurin-dependent inactivation



**Figure 1. CDI of neuronal L-type Ca<sup>2+</sup> channels requires AKAP79/150-anchored PKA**  
 (A) (Left) Top three images, fluorescence images of cultured rat neurons transfected with 150RNAi and: wild-type AKAP79 (79wt), AKAP79 PKA (79 PKA), or AKAP79Pro2 (79Pro2). Bottom three images, differential interference contrast images of short-term cultured neurons from wild-type mice, AKAP150 PKA mice and AKAP150<sup>-/-</sup> mice. (Center) Superimposed, normalized records of pharmacologically-isolated L-channel Ca<sup>2+</sup> (red) or Ba<sup>2+</sup> (black) currents. (Right) Mean inactivation rates ( $1/\tau$ ). Number of cells recorded per condition (*n*) is noted on each bar.  
 (B) CDI index, calculated as the difference between  $r_{400}$  in Ba<sup>2+</sup> and  $r_{400}$  in Ca<sup>2+</sup>. Black bars represent knockdown/replacement experiments in rat neurons and gray bars represent knockin mice, in all four panels. Fractional depression of divalent charge influx as a consequence of CDI, calculated as the difference between peak-normalized Ba<sup>2+</sup> charge influx and peak-normalized Ca<sup>2+</sup> charge influx during 500 ms depolarizations.  
 (C) Peak Ca<sup>2+</sup> current density and Ca<sup>2+</sup> charge density for each of the experimental manipulations in B. Dividing peak Ca<sup>2+</sup> current ( $I_{Ca}$ , pA) by a measure of membrane surface area, cell capacitance ( $C_m$ , pF), yields the density of Ca<sup>2+</sup> current (pA/pF). Ca<sup>2+</sup> charge

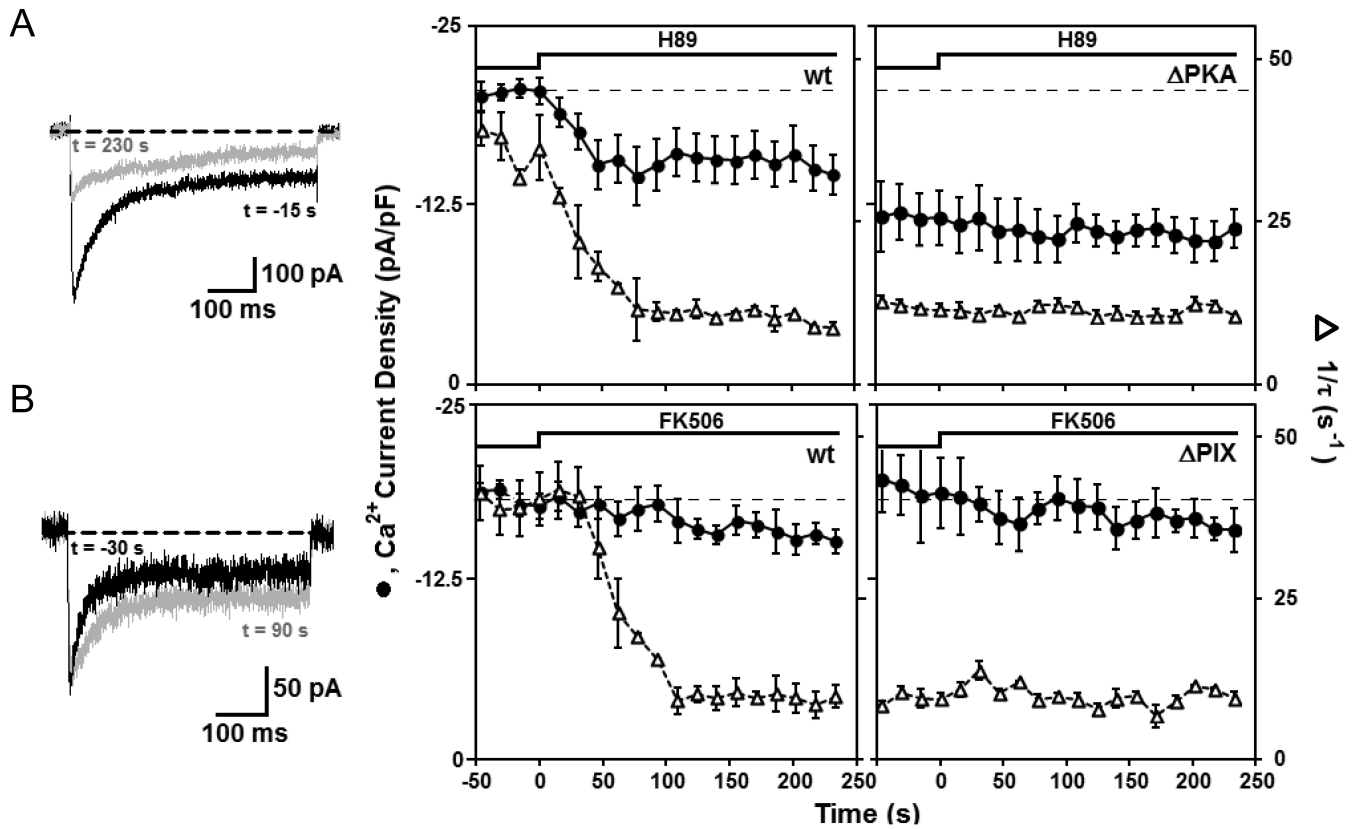
density (pC/pF) was calculated as the integrated  $\text{Ca}^{2+}$  current during 500-ms depolarization (pC) divided by cell capacitance (pF).

(D) For 150RNAi-transfected rat neurons, voltage-dependence of  $\text{Ca}^{2+}$  current density for 79wt (●) or 79 PKA (▲) co-transfected neurons, compared with the voltage-dependence of inactivation rate for  $\text{Ca}^{2+}$  current, for 79wt (○) or 79 PKA (△) co-transfected neurons. Error bars indicate standard error of the mean. Mean values were compared using ANOVA with a posthoc Bonferroni correction. Statistical significance marked as \*  $p = 0.05$ , \*\* $p = 0.01$ , \*\*\*  $p = 0.005$ , and \*\*\*\*  $p = 0.001$ .



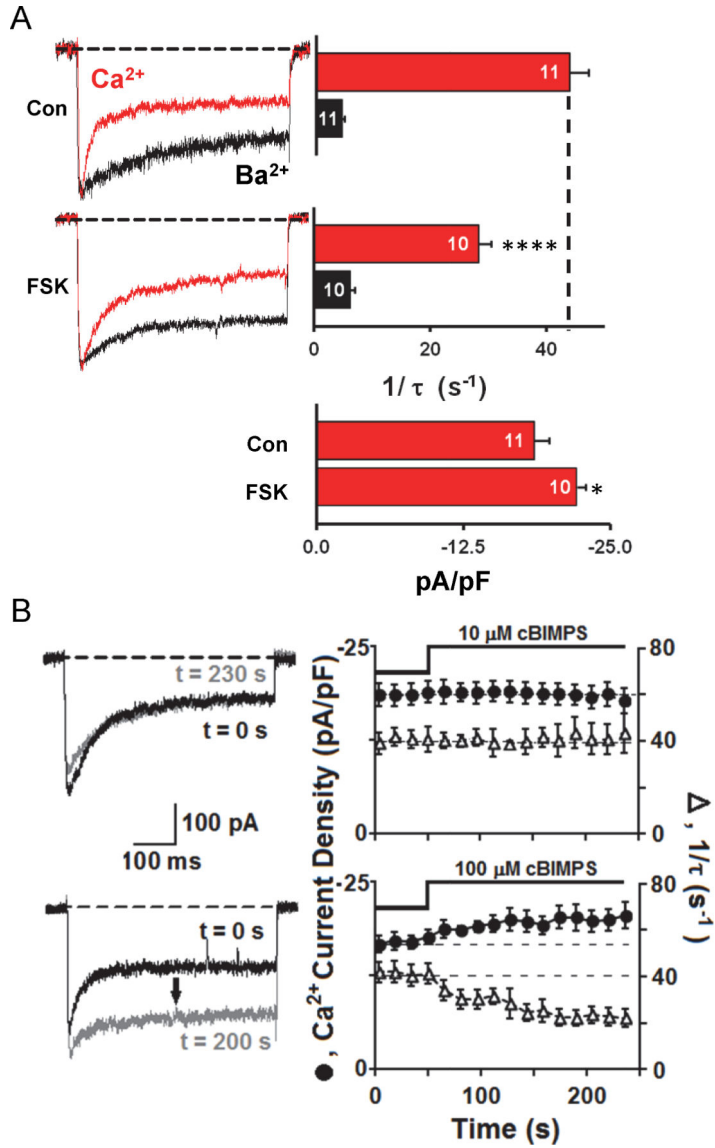
**Figure 2. CDI of neuronal L-type  $Ca^{2+}$  channels is suppressed by acute inhibition of PKA anchoring or activity**

(A) Ht31 peptide, a competitive inhibitor of PKA anchoring to AKAP150, decreased CDI rate and current density of L-type  $Ca^{2+}$  channels in short-term cultured hippocampal neurons. Ht31 peptide was perfused into the cell through the patch pipet. (Left) Normalized  $Ca^{2+}$  (red) and  $Ba^{2+}$  currents (black), recorded from the same cell, were evoked by 500-ms step-depolarization from  $-60$  to  $0$  mV. (Right) Inactivation rates for  $Ca^{2+}$  current (red bars) and  $Ba^{2+}$  current (black bars). Control rate measured with 0.1% DMSO in the patch. Incorporation of Ht31-Pro in place of Ht31 in the patch pipet had no effect on current density or CDI. (B) Dose-response relationships for neurons treated with Ht31, (●) current density and (△)  $1/\tau$ . (C) Inhibition of PKA activity by internal perfusion with  $5$   $\mu$ M PKI suppresses CDI and lowers current density. Error bars indicate standard error of the mean. Mean values were compared using ANOVA with a posthoc Bonferroni correction. Statistical significance marked as \*\*\*\*  $p = 0.001$ .



**Figure 3. Time course of changes in current density and inactivation rate induced by inhibition of PKA or CaN**

(A) Response to fast perfusion of H89 (500 nM). (*Left*) Untransfected rat neurons. Inset, superimposed  $\text{Ca}^{2+}$  current records before (*black*) and during (*gray*) H89 application. (*Right*) Rat neurons expressing 79 PKA. (B) Response to fast application of FK506 (5  $\mu$ M). (*Left*) Untransfected rat neurons. Inset, representative  $\text{Ca}^{2+}$  current records before (*black*) and during (*gray*) FK506 application. (*Right*) Rat neurons expressing 79 PIX. Error bars represent standard error of the mean.



**Figure 4. CDI of neuronal L-type Ca<sup>2+</sup> channels is reduced by PKA pathway activation**  
 (A) Internal perfusion with forskolin slows Ca<sup>2+</sup>-dependent inactivation of L-type current and increases current density. Peak Ca<sup>2+</sup> current amplitude (red) was normalized to peak Ba<sup>2+</sup> current (black) amplitude. Bar graphs present inactivation rates for Ca<sup>2+</sup> and Ba<sup>2+</sup> currents; number of individual cells recorded (n) is marked on each bar.  
 (B) Bath application of 100  $\mu$ M cAMP analog (Sp-5,6-dichloro-cBIMPS) (bottom), but not 10  $\mu$ M Sp-5,6-dichloro-cBIMPS (top), enhances current density and slows Ca<sup>2+</sup>-dependent inactivation. Error bars indicate standard error of the mean. Mean values were compared using ANOVA with a Bonferroni posthoc correction. Statistical significance marked as \* p = 0.05, \*\*\*\* p = 0.001.

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