Ca\(^{2+}\) Controls Functional Expression of the Cardiac K\(^{+}\) Transient Outward Current via the Calcineurin Pathway*

Emeline Perrier, Romain Perrier, Sylvain Richard, and Jean-Pierre Bénitah‡

From the INSERM U637, CHU A. de Villeneuve, 34295 Montpellier, France

The transient outward K\(^{+}\) current (I\(_{to}\)) modulates transmembrane Ca\(^{2+}\) influx into cardiomyocytes, which, in turn, might act on I\(_{to}\). Here, we investigated whether Ca\(^{2+}\) modifies functional expression of I\(_{to}\). Whole-cell I\(_{to}\) were recorded using the patch clamp technique in single right ventricular myocytes isolated from adult rats and incubated for 24 h at 37 °C in a serum-free medium containing various Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{o}\)]. Increasing the [Ca\(^{2+}\)\(_{o}\)] from 0.5 to 1.0 and 2.5 mM produced a gradual decrease in I\(_{to}\) density without change in current kinetics. Quantitative reverse transcriptase-PCR showed that a decrease of the Kv4.2 channel mRNA could account for this decrease. In the acetoxymercaptopropionylglycerol (W7) or the calcineurin inhibitors FK506 or cyclosporin A. Furthermore, in myocytes incubated for 24 h with 2.5 mM [Ca\(^{2+}\)\(_{o}\)], calcineurin activity was significantly increased compared with 1 mM [Ca\(^{2+}\)\(_{o}\)]. Our data suggest that modulation of [Ca\(^{2+}\)\(_{o}\)] via L-type Ca\(^{2+}\) channels, which appear to involve the Ca\(^{2+}\)-calmodulin-regulated protein phosphatase calcineurin, down-regulates the functional expression of I\(_{to}\). This effect might be involved in many physiological and pathological modulations of I\(_{to}\) channel expression in cardiac cells, as well other cell types.

K\(^{+}\) channels play critical roles in a wide variety of physiological processes including regulation of heart rate and contraction. Among other cardiac K\(^{+}\) currents, the transient outward K\(^{+}\) current (I\(_{to}\)) is crucial because it controls the amplitude of the plateau phase and the duration of the action potential. As a consequence, I\(_{to}\) strongly modulates transmembrane Ca\(^{2+}\) entry and, thereby, the excitation-contraction coupling (1). Thus, any change in I\(_{to}\) has profound pathophysiological consequences, often leading to the generation of life-threatening arrhythmias. For example, changes in the density and/or the properties of I\(_{to}\) occur in conjunction with myocardial damage or disease, including acute or chronic diabetes mellitus, hypertrophy induced by pressure or volume overload, and cardiac failure (2). Unidentified common pathway(s) may underlie a down-regulation of the expression of I\(_{to}\) channels in all these pathological conditions. A possible candidate for triggering such channel remodeling could be the altered level of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{i}\)]).

Even if defined as Ca\(^{2+}\)-independent, by contrast to the 4-aminopyridine-resistant Ca\(^{2+}\)-activated transient outward current, I\(_{to}\) might be regulated by Ca\(^{2+}\). A modulation of I\(_{to}\) by extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{o}\)]) induced by depolarizing shifts in the gating parameters has been documented (3). Moreover, I\(_{to}\) inactivation in human atrial myocytes might be controlled by [Ca\(^{2+}\)]-dependent processes, involving Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) (4). At the molecular level, shal-type voltage-gated K\(^{+}\) channels (in rat predominantly Kv4.2 (5)) are the pore-forming subunits of the I\(_{to}\) channel that are regulated by auxiliary subunits (5, 6). Interestingly, the regulatory subunits KChIPs and the neuronal calcium sensor (NCS) that modulate I\(_{to}\) expression and kinetics are Ca\(^{2+}\)-binding proteins. Thus, Ca\(^{2+}\) controls the gating of Kv4-KChIP (7) or Kv4-NCS-1 complexes (8). In addition to these acute effects, Ca\(^{2+}\) also affects cardiac gene expression through Ca\(^{2+}\)-dependent transcription factors. For example, Ca\(^{2+}\)-sensitive transcription factors may play a key role in maintaining the contractile phenotype and contribute to adaptive or pathological changes in the structural or functional properties of the heart (9). Notably, long-term changes in intracellular Ca\(^{2+}\) level have been linked to altered functional expression of the ion channel. In cultured neonatal cardiomyocytes, increases in [Ca\(^{2+}\)\(_{i}\)] cause a fall in density of the Na\(^{+}\) current (11). Exposure of adult rat ventricular myocytes in culture to high Ca\(^{2+}\) increases Ca\(^{2+}\) channel mRNA and protein abundance, producing a corresponding change in the L-type Ca\(^{2+}\) channel (I\(_{Ca}\)) (12).

In the present study, we hypothesized that Ca\(^{2+}\) regulates the functional expression of I\(_{to}\). Experimental manipulations of Ca\(^{2+}\) entry via I\(_{Ca}\) in adult rat ventricular myocytes incubated for 24 h, showed that increased cytosolic Ca\(^{2+}\) decreases I\(_{to}\) and down-regulates Kv4.2 mRNA expression, which involves Ca\(^{2+}\)-calmodulin-regulated protein phosphatase calcineurin cascade.

**Experimental Procedures**

**Cell Isolation and Incubation**—Cardiac ventricular myocytes were isolated from adult male Wistar rats (250–280 g) using an enzymatic

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1 To whom correspondence should be addressed: INSERM U637, CHU A. de Villeneuve, 34295 Montpellier, France. Tel.: 33-4-67-41-52-38; Fax: 33-4-67-41-52-42; E-mail: benitah@montp.inserm.fr.
Cells incubated with 1 mM [Ca\(^{2+}\)] were normalized to the amount of mRNA coding for cyclophilin present in the same cell extracts and then expressed as percentage of ratio observed in controls.

Amplitude histograms show the relative expression of Kv4.2 gene mRNA in isolated myocytes incubated 24 h with 0.5, 1, or 2.5 mM [Ca\(^{2+}\)]. Data were normalized to the amount of mRNA coding for cyclophilin present in the same cell extracts and then expressed as percentage of ratio observed in cells incubated with 1 mM [Ca\(^{2+}\)]. Columns represent mean values of n experiments. *, p < 0.05; **, p < 0.005; and ***, p < 0.0005 versus 1 mM [Ca\(^{2+}\)].

**RESULTS**

**High Extracellular Ca\(^{2+}\) Decreases I\(_{to}\) and Kv4.2 mRNA Expression**—Fig. 1A, left, shows typical examples of the 4-aminopyridine-sensitive I\(_{to}\) recorded in myocytes incubated for 24 h at three different [Ca\(^{2+}\)]. I\(_{to}\) density showed a gradual decrease correlating with [Ca\(^{2+}\)] increase from 0.5 to 1.0 and 2.5 mM. On average, the current density-voltage relationships show statistically significant variations for all voltages above +10 mV depending on the [Ca\(^{2+}\)]. Detailed analysis revealed that these variations were not related to changes in voltage- or time-dependent properties of I\(_{to}\) during the 24-h incubation (Table I).

**Calcineurin Activity**—Cellular calcineurin phosphatase activity was measured on cell extract using the Calbiochem Calcineurin Cellular Activity Assay Kit according to the manufacturer’s instructions. The fraction of total phosphatase activity due to calcineurin was determined by detection of free phosphate released in absence or presence of EGTA buffer. Colorimetric measure (assay with Malachite Green) was done at 620 nm on a plate reader (Dynatech MR 5000). [Ca\(^{2+}\)], Measurement—After 24-h incubation, cells were loaded with 2.5 mM Fura-2 AM (Molecular Probes) for 30 min at 37 °C. Cells were then rinsed in respective storage solution (1 or 2.5 mM [Ca\(^{2+}\)]), and fluorescence measurements were made using the MetaFlour imaging system (Universal Imaging Corp.). [Ca\(^{2+}\)] was calculated as reported previously (11); the used dissociation constant of Fura-2 for Ca\(^{2+}\) was 0.141 μM.

**Statistical Analysis**—Data were presented as mean ± S.E. Unpaired Student’s t test was used for comparisons. A value of p < 0.05 was accepted as statistically significant.

**DISCUSSION**

The results show that high extracellular Ca\(^{2+}\) decreases I\(_{to}\) and Kv4.2 mRNA expression in cardiac myocytes. The decrease in I\(_{to}\) density is likely due to a decrease in Kv4.2 mRNA expression, as suggested by the decrease in calcineurin activity. The decrease in calcineurin activity may be due to the increase in [Ca\(^{2+}\)].
Ca²⁺ Controls \( I_{lo} \) Expression

The voltage-dependent availability were fit to a Boltzmann distribution with \( E_{inac} \), the potential of half-inactivation, and \( k \), the slope factor. The time to peak values, \( T_{peak} \), were determined as the time from the onset of depolarization at +40 mV to the time of maximal current amplitude and reflect activation rates. The inactivation kinetics were determined by fitting the decay phase of the current traces to a monoexponential function with \( \tau_{inac} \), time constant at +40 mV. 

### Table I

| \( [Ca^{2+}]_o \) | \( E_{inac} \) | \( k \) | \( \tau_{inac} \) | \( T_{peak} \) |
|------------------|-------------|-------|-----------------|-------------|
| 0.5 mM [Ca²⁺]ₙ | 149.3 ± 10.6 | -11.6 ± 0.3 | 6.9 ± 0.3 | 10.7 ± 0.5 | 21.7 ± 1.2 |
| 1 mM [Ca²⁺]ₙ | 147.1 ± 14.8 | -10.6 ± 0.4 | 7.0 ± 0.4 | 11.5 ± 0.8 | 25.1 ± 1.1 |
| +10 μM BAPTA-AM | 149.2 ± 11.3 | -11.7 ± 0.6 | 6.5 ± 0.6 | 10.1 ± 0.4 | 25.4 ± 1.5 |
| +1 μM Bay K8644 | 148.9 ± 13.7 | -10.3 ± 0.3 | 7.1 ± 0.3 | 11.5 ± 0.7 | 27.0 ± 1.8 |
| 2.5 mM [Ca²⁺]ₙ | 142.8 ± 12.7 | -10.4 ± 0.6 | 7.0 ± 0.5 | 10.9 ± 0.6 | 27.5 ± 1.3 |
| +10 μM BAPTA-AM | 141.8 ± 14.3 | -10.6 ± 0.7 | 6.8 ± 0.6 | 9.7 ± 0.3 | 29.2 ± 1.9 |
| +0.1 μM nifedipine | 148.5 ± 13.2 | -10.6 ± 0.3 | 6.6 ± 0.3 | 10.2 ± 0.3 | 26.7 ± 1.0 |
| +1 μM W7 | 141.5 ± 10.1 | -10.2 ± 0.4 | 6.9 ± 0.3 | 11.8 ± 0.4 | 24.7 ± 1.0 |
| +1 μM KN-62 | 148.4 ± 11.7 | -9.9 ± 0.4 | 6.5 ± 0.4 | 11.7 ± 0.5 | 27.6 ± 1.5 |
| +1 μM FK506 | 140.9 ± 7.5 | -11.5 ± 0.4 | 6.9 ± 0.4 | 10.7 ± 0.5 | 28.2 ± 1.2 |
| +25 μM CsA | 140.1 ± 6.4 | -11.2 ± 0.4 | 7.3 ± 0.4 | 11.8 ± 0.6 | 29.1 ± 1.5 |

**Fig. 2.** Decrease in \( I_{lo} \) density is mediated by increase [Ca²⁺]ₙ through \( I_{lo} \). Pattern of \( I_{lo} \) and bar graphs of pooled \( I_{lo} \) slope conductance (\( G_{lo} \)) in myocytes incubated 24 h with 1 mM [Ca²⁺]ₙ (left) in the absence of drugs or co-incubated with either a membrane-permeant Ca²⁺ chelator (BAPTA-AM) or an \( I_{lo} \) agonist (Bay K8644) and with 2.5 mM [Ca²⁺]ₙ (right) in the absence of drugs or co-incubated with either BAPTA-AM or an \( I_{lo} \) antagonist (Nifedipine). The \( G_{lo} \)-voltage relationships (from +10 to +60 mV) under each condition were fitted with a linear function to estimate \( G_{lo} \), number of experiments. \( * \), \( p < 0.05 \) versus 1 mM [Ca²⁺]ₙ; \( \# \), \( p < 0.05 \) versus 2.5 mM [Ca²⁺]ₙ.

We next investigated the expression level of Kv4.2, quantified by real-time RT-PCR after 24-h incubation of the cells at the three different [Ca²⁺]ₙ. When normalized to the amount of cyclophilin mRNA present in the same cell extracts, we observed a gradual decrease of Kv4.2 mRNA expression (Fig. 1B). This did not exclude changes in other message levels, such as Kv4.3 or KChIP. Overall, these experiments suggested that the high [Ca²⁺]ₙ-induced decrease in \( I_{lo} \) density correlates with a...
Ca\textsuperscript{2+} Controls \(I_{to}\) Expression

**Correspondence:**

**Yin Y. Zhang**

**Department of Molecular and Cellular Physiology**

**Stanford University, Stanford, California 94305**

**E-mail:**

yinzhang@stanford.edu

The calmodulin/calcineurin-signaling pathway is involved in Ca\textsuperscript{2+}-dependent decrease of \(I_{to}\). A. Representative examples of \(I_{to}\) families and an amplitude histogram of pooled \(G_{s,lo}\) in myocytes incubated 24 h with 2.5 mM [Ca\textsuperscript{2+}]. In the absence of drugs or co-incubated with either a Ca\textsuperscript{2+}-calmodulin complex inhibitor (W7), a calmodulin kinase II inhibitor (KN-62), or calcineurin inhibitors (FK506 or CsA). Values are expressed in percentage of \(G_{s,lo}\) of cells incubated with 1 mM [Ca\textsuperscript{2+}]. B. 24-h incubation with increased [Ca\textsuperscript{2+}] produced calcineurin activity increase. Bar graph of calcineurin activity, represented by the amount of phosphate released, in myocytes incubated 24 h at 37°C with either 1 or 2.5 mM [Ca\textsuperscript{2+}] is shown. **, \(p<0.005\) and ***, \(p<0.0005\) versus 2.5 mM [Ca\textsuperscript{2+}]..

decrease in Kv4.2 mRNA expression and, therefore, seems to involve gene regulation.

**Intracellular Ca\textsuperscript{2+} Buffering Prevents Down-regulation of \(I_{to}\).** Increased [Ca\textsuperscript{2+}], is expected to cause an increase in cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]). To check it, we evaluated the mean diastolic [Ca\textsuperscript{2+}] using Fura-2 AM in myocytes incubated 24 h. [Ca\textsuperscript{2+}], was significantly (\(p<0.005\)) higher in cells incubated with 2.5 mM [Ca\textsuperscript{2+}], (228.8 ± 3.1 nm (n = 27)) than with 1 mM [Ca\textsuperscript{2+}], (100.7 ± 1.1 nm (n = 24)). Then we repeated our electrophysiological experiments on cells incubated 24 h in either 1 or 2.5 mM [Ca\textsuperscript{2+}] in the presence of the membrane-permeant calcium chelator, BAPTA-AM. As shown in Fig. 2, 10 \(\mu\text{M}\) BAPTA-AM not only increased \(I_{to}\) in cells exposed to 1 mM [Ca\textsuperscript{2+}], but also blunted the decrease expected at 2.5 mM [Ca\textsuperscript{2+}], (Fig. 1). Thus, decreased [Ca\textsuperscript{2+}], resulted in a significant increase of \(I_{to}\) slope conductance.

**Ca\textsuperscript{2+} Channels Mediate Ca\textsuperscript{2+}-dependent Down-regulation of \(I_{to}\).** The mechanisms by which [Ca\textsuperscript{2+}] promotes an increase in [Ca\textsuperscript{2+}], may involve greater leak across the sarcolemma through L-type Ca\textsuperscript{2+} channels or stimulation of the Na/Ca\textsuperscript{2+} exchanger working in the “reverse mode” (17). Even if small, the open probability of the L-type Ca\textsuperscript{2+} channel at rest is not null as assessed by single channel recording (18). Moreover, increasing the driving force across the membrane in presence of higher [Ca\textsuperscript{2+}], will enhance channel conductance (19). We measured \(I_{to}\) in myocytes incubated for 24 h with either a Ca\textsuperscript{2+} channel agonist (Bay K8644) or a Ca\textsuperscript{2+} channel antagonist (Fig. 2, +Nifedipine). Incubation with 1 \(\mu\text{M}\) Bay K8644 in the presence of 1 mM [Ca\textsuperscript{2+}], decreased \(I_{to}\) thereby mimicking the effect of incubation at high [Ca\textsuperscript{2+}], (2.5 mM). In contrast, 24-h incubation with nifedipine (0.1 \(\mu\text{M}\)) blunted the decrease of \(I_{to}\) observed at high [Ca\textsuperscript{2+}] (Fig. 2). It is worth noting that none of those treatments altered \(I_{to}\) kinetics and voltage dependence (Table I).

These experiments suggested, therefore, that down-regulation of the functional expression of \(I_{to}\) occurs mostly by modulation of [Ca\textsuperscript{2+}], through L-type calcium current.

**Ca\textsuperscript{2+}-induced Down-regulation of \(I_{to}\) Involves the Calcineurin Pathway.** Many of the actions of Ca\textsuperscript{2+} are mediated through its interaction with CaM. CaM serves as an intracellular sensor for Ca\textsuperscript{2+} and selectively activates specific downstream signaling pathways in response to local changes in [Ca\textsuperscript{2+}], (20). To focus on the Ca\textsuperscript{2+}-triggered pathway involved in the down-regulation of \(I_{to}\) one group of cells was treated with the Ca\textsuperscript{2+}/CaM inhibitor W7 (21). The effect of 1 \(\mu\text{M}\) W7 in the presence of 2.5 mM [Ca\textsuperscript{2+}], is shown in Fig. 3. W7 prevented the down-regulation of \(I_{to}\) induced by high Ca\textsuperscript{2+}, with no change in the kinetics and in the voltage dependence of \(I_{to}\) (Table I). This finding suggested that Ca\textsuperscript{2+} modulation of \(I_{to}\) involves CaM.

Two Ca\textsuperscript{2+}/CaM-dependent enzymes that have major effects on cardiac muscle function are the CaMKII (22) and the phosphatase calcineurin (23). These enzymes have distinct Ca\textsuperscript{2+} sensitivities, partner proteins, and subcellular localizations that enable them to discriminate between different types of Ca\textsuperscript{2+} signals and regulate different functions (9).
To characterize further the Ca2⁺ pathway for long term regulation of I_{\text{to}}, we used the CaMKII inhibitor KN-62 that has been reported to specifically inhibit Ca2⁺/CaM protein kinase isofoms (24). Exposure of cells to 1 μM KN-62 in 2.5 mM [Ca2⁺]_o for 24 h did not prevent the down-regulation of I_{\text{to}} (Fig 3A). In contrast, incubation with tacrolimus (FK506, 1 μM) or CsA (25 μM), known to block calcineurin (25), prevented I_{\text{to}} down-regulation (Fig. 3A). Neither the time- nor the voltage-dependent properties of I_{\text{to}} were changed after these treatments (Table I).

Exposure of cardiomyocytes to higher doses of W7 (26), FK506, or CsA (27) might exert direct acute effects on I_{\text{to}} and/or I_{\text{ca}}. To confirm calcineurin involvement, we checked its activity in myocytes incubated for 24 h in either 1 or 2.5 mM [Ca2⁺]_o using a colorimetric assay. Calcineurin activity determined by the amount of phosphate release was increased significantly in 2.5 mM [Ca2⁺]_o-treated cells (Fig. 3B), suggesting that enhancement of calcineurin activity after Ca2⁺ treatment mediates I_{\text{to}} down-regulation. We concluded, therefore, that the Ca2⁺/CaM-regulated protein phosphatase, calcineurin, is involved in the long term Ca²⁺-dependent down-regulation of I_{\text{to}}.

**DISCUSSION**

The present study shows that increased Ca²⁺ influx through L-type Ca²⁺ channels causes an increase in [Ca²⁺]_i, which up-regulates the Ca²⁺/calcineurin pathway and leads to a down-regulation of the expression of I_{\text{to}} (Kv4.2 gene) in isolated adult rat ventricular myocytes. Fig. 4 summarizes our data.

Long term changes in cardiac cellular excitability can be generated by regulating the expression of I_{\text{to}} channel genes (1, 5, 6, 28). This was well demonstrated by use of transgenic and targeted deletion strategies in mice (29). On the other hand, electrical activity might affect K⁺ channel gene expression. It has been reported that membrane depolarization or Bay K8644 (30) enhances expression of the rapid inactivating Shaker K⁺ channel Kv1.4 mRNA related to I_{\text{to}} in newborn rat cardiomyocytes (31). Chronic membrane depolarization of cultured neonatal myocytes reduces I_{\text{to}} density without affecting either current kinetics or voltage dependence (32). Our data suggest that Ca²⁺ influx through voltage-dependent Ca²⁺ channels is a route for modulation of functional expression of I_{\text{to}}. Indeed, the Ca²⁺ channel agonist Bay K8644 mimics the effect of high [Ca²⁺]_o on I_{\text{to}} (decrease), whereas the Ca²⁺ channel antagonist nifedipine has the opposite effect (increase) analogous to the effect of reducing [Ca²⁺]_o.

We demonstrate here that the calmodulin inhibitor, W7, blunts the decrease in I_{\text{to}} at high [Ca²⁺]_o. An emerging body of work indicates that the Ca²⁺-calmodulin complex acts as an important second messenger for various signals, including angiotensin II, endothelin-1, α-adrenergic agents, and a mechanical stretch that triggers hypertrophic growth of the myocardium (33, 34). Interestingly, these modulators regulate both Kv4 channel current densities and mRNA levels. For example, angiotensin II has been implicated in the modulation of I_{\text{to}} (35). An autocrine/paracrine release of ET-1 modulates Kv4.2 and Kv1.2 (35). Thyroid status also regulates I_{\text{to}} density and Kv4.2 mRNA levels (36). Thus, some of the effects on I_{\text{to}} in response to a variety of endogenous modulators and hormones known, or postulated, to modulate cardiac function might involve changes in Ca²⁺ signaling, in particular, related to I_{\text{ca}} regulation. In support of this view, we recently demonstrated that aldosterone-induced downregulation of I_{\text{to}} functional expression occurs secondary to modulation of Ca²⁺ signaling (increased I_{\text{ca}}) (13).

Members of the diverse superfamily of voltage-activated K⁺ channels are modulated by phosphorylation. Such modulations are catalyzed by a variety of protein kinases including CaMKII. In atrial myocytes, phosphorylation of the K⁺ channel by CaMKII affects the fast inactivation kinetics (4). In the present study we did not observe either kinetic modification of I_{\text{to}} with
Numerous studies indicate that alterations in intracellular 
from extracellular signals received by animal cells to intracellular one of the main routes by which information is transferred 
response (41, 42). As reported, the Ca$^{2+}$ pipette that will attenuate acute intracellular Ca$^{2+}$
density after myocardial infarction (38). Moreover, in contrast to a 
that, in this pathological condition, increased diastolic 
I$\text{to}$, CsA at low concentrations prevents Ca$^{2+}$ down-regulation of I$\text{to}$ at high [Ca$^{2+}$].

Changes in the cytoplasmic free Ca$^{2+}$ concentration constitute 
one of the main routes by which information is transferred from extracellular signals received by animal cells to intracellular sites. In heart, these changes have been implicated in regulating diverse physiological and pathological processes. Numerous studies indicate that alterations in intracellular Ca$^{2+}$ signaling are a primary stimulus for the hypertrophic response (41, 42). As reported, the Ca$^{2+}$/calmodulin-activated phosphatase calcineurin and its downstream transcriptional effector calcineurin-nuclear factor of activated T-cells (NFAT) have been implicated as transducers of the pathological hypertrophic response (43). Importantly, decreased I$\text{to}$ and down-regulated expression of ventricular Kv4 mRNAs are consistent findings in the hypertrophic myocardium, which can lead to increases in Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels during longer action potential (44). On the other hand, we suggest that, in this pathological condition, increased diastolic [Ca$^{2+}$], might induce down-regulation of I$\text{to}$. Thus, Ca$^{2+}$ influx is under control I$\text{to}$ through action potential duration influence, resulting in elevated intracellular Ca$^{2+}$ levels, which in turn regulates I$\text{to}$ and then reshapes action potential waveforms favoring Ca$^{2+}$ influx. We summarize this mechanism as Ca$^{2+}$-induced Ca$^{2+}$ entry. More generally, our finding, if extended to other cell types, might help to understand how cells coordinate the expression of K$^{+}$ channel for regulation of excitability.

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