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SYMPOSIUM REVIEW

# Role of voltage-gated calcium channels in the regulation of aldosterone production from zona glomerulosa cells of the adrenal cortex

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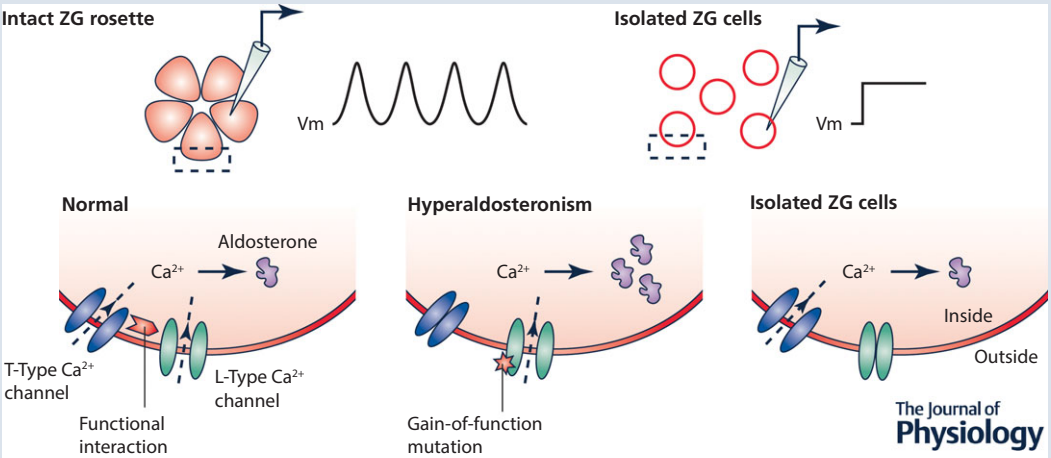


**Abstract** Zona glomerulosa cells (ZG) of the adrenal gland constantly integrate fluctuating ionic, hormonal and paracrine signals to control the synthesis and secretion of aldosterone. These signals modulate $Ca^{2+}$ levels, which provide the critical second messenger to drive steroid hormone production. Angiotensin II is a hormone known to modulate the activity of voltage-dependent L- and T-type $Ca^{2+}$ channels that are expressed on the plasma membrane of ZG cells in many species. Because the ZG cell maintains a resting membrane voltage of approximately −85 mV and has been considered electrically silent, low voltage-activated T-type $Ca^{2+}$ channels are assumed to provide the primary $Ca^{2+}$ signal that drives aldosterone production. However, this view has recently been challenged by human genetic studies identifying somatic gain-of-function mutations in L-type $Ca_V1.3$ channels in aldosterone-producing adenomas of patients with

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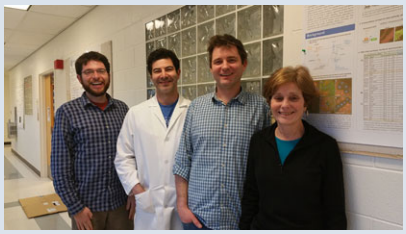

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primary hyperaldosteronism. We provide a review of these assumptions and challenges, and update our understanding of the state of the ZG cell in a layer in which native cellular associations are preserved. This updated view of $Ca^{2+}$ signalling in ZG cells provides a unifying mechanism that explains how transiently activating $Ca_V3.2$ channels can generate a significant and recurring $Ca^{2+}$ signal, and how $Ca_V1.3$ channels may contribute to the $Ca^{2+}$ signal that drives aldosterone production.

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**Abstract figure legend** In the intact rosette, ZG cells are electrically excitable, a behavior that is not evident when cellular associations are disrupted. In the electrically quiescent ZG cell (isolated) T-channel activity is favored and controls aldosterone output. In health (normal), T-channel activity recruits L-channel opening and together their activities regulate intracellular calcium to sustain the production of aldosterone. In disease (primary hyperaldosteronism) functional mutations in L-channels promote their dominant control.

**Abbreviations** APA, aldosterone producing adenoma; CDI, $Ca^{2+}$-dependent inactivation; IHA, idiopathic bilateral zona glomerulosa hyperplasia; PA, primary hyperaldosteronism; RAS, renin–angiotensin system; ZG, zona glomerulosa.

## Primary hyperaldosteronism

Circulating aldosterone is synthesized and secreted by zona glomerulosa (ZG) cells of the adrenal cortex. When under the control of the renin–angiotensin system (RAS), aldosterone production is commensurate with the level of circulating renin. By promoting $Na^+$ retention at multiple sites along the nephron, aldosterone contributes to the physiological regulation of electrolytes and water balance. Excessive aldosterone production independent of the RAS can occur, as in primary hyperaldosteronism (PA), and is characterized by a large increase in the aldosterone/renin ratio (Montori & Young, 2002). Because aldosterone over-production in PA has additional adverse effects on the heart (Catena *et al.* 2008) and kidneys (Rossi *et al.* 2006) that are independent of blood pressure elevation, this most common type of endocrine hypertension is associated with increased cardiovascular risk (Stowasser, 2001; Savard *et al.* 2013). The most common forms of PA result from two major pathologies: unilateral aldosterone producing adenoma (APA) and idiopathic bilateral zona glomerulosa hyperplasia (IHA). With a prevalence of only 4% in the general population, the incidence of PA rises significantly among hypertensive patients ($>$ 10%) (Plouin *et al.* 2004; Rossi *et al.* 2006) and markedly among those with resistant hypertension (20%) (Calhoun *et al.* 2002*a*,*b*; Stowasser, 2014).

## Pathogenic mechanisms: insights from human genetic analysis of channels and pumps

Since 2011, great progress has been made in our understanding of the genetic bases for APA. Choi *et al.* (2011) were the first group to identify recurrent somatic mutations in the coding region of the *KCNJ5* G-protein coupled, inwardly rectifying $K^+$ channel in DNA extracted from aldosterone producing adenomas. These mutations occur in or near the selectivity filter of the channel rendering it permeable to $Na^+$. Originally identified in a small cohort of 22 severely hypertensive patients, these or functionally equivalent mutations in exon 2 of the *KCNJ5* gene are now the most frequently identified genetic variants (∼38%) in aldosterone-producing adenomas (Azizan *et al.* 2012; Boulkroun *et al.* 2012; Mulatero *et al.* 2012; Dekkers *et al.* 2014; Fernandes-Rosa *et al.* 2014; Kuppusamy *et al.* 2014). Less frequent variants have been identified in the P-type ATPase gene family, *ATP1A1* and *ATP2B3*, encoding $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase3, respectively (∼7%) (Azizan *et al.* 2013; Beuschlein *et al.* 2013; Fernandes-Rosa *et al.* 2014). Mutations residing in transmembrane helices M1 and M4 of the $Na^+/K^+$-ATPase cause loss of functional pump activity, the appearance of new ouabain-insensitive $Na^+$ or $H^+$ inward leak currents and cell depolarization (Azizan *et al.* 2013; Beuschlein *et al.* 2013). Cell depolarization is also induced by mutations in the $Ca^{2+}$-ATPase3 found in the M4 transmembrane helix that forms the $Ca^{2+}$ binding pocket of the $Ca^{2+}$ pump (Beuschlein *et al.* 2013). By contrast, multiple and more frequent (5–14%) somatic mutations have been identified in the *CACNA1D* gene encoding the $Ca_V1.3$ voltage-dependent $Ca^{2+}$ channel (Azizan *et al.* 2013; Scholl *et al.* 2013; Fernandes-Rosa *et al.* 2014). These variants are scattered broadly throughout the large $\alpha1$ subunit of the channel protein, and are found in the activation gate (S6 helices), the voltage sensor (S4 helices) and in the S4-S5 cytoplasmic linkers connecting the voltage sensor to the channel pore. In general, these mutations result in a gain of $Ca^{2+}$ channel function, increasing the probability for channel opening at hyperpolarized potentials, either alone or in combination with a shift in the voltage dependence of channel inactivation. Some mutations also dramatically slow the time dependence

of inactivation. In addition, several germline mutations in the *KCNJ5* gene outside the selectivity filter were recently described in patients with apparent sporadic PA (Murthy *et al.* 2014), and a rare recurrent mutation in the *CACNA1H* gene encoding the $Ca_V3.2$ voltage-dependent $Ca^{2+}$ channel was identified in children presenting with severe hyperaldosteronism and hypertension (Scholl *et al.* 2015). The latter mutation found in the IIIS6 domain markedly slows the time dependence of inactivation of this rapidly inactivating channel without altering the voltage dependencies of channel gating.

Interestingly, the mutational status of tumours among different patient cohorts does not support the hypothesis that APA is associated with a consistent histological phenotype; mutations do not segregate according to cell type (zona glomerulosa, zona fasciculata or mixed). Neither was there a consistent molecular signature (e.g. the overexpression of *CYP11B2*, *KCNK5*, *CACNA1D*, *ATP1A1* genes) (Enberg *et al.* 2004; Lenzini *et al.* 2007; Fernandes-Rosa *et al.* 2014; Boulkroun *et al.* 2015). One potential exception may include the *CACNA1H* gene whose level of mRNA expression significantly associates with peripheral blood aldosterone levels, *CYP11B2* gene expression levels and *KCNJ5* mutational status in a cohort of 74 Japanese APA patients (Felizola *et al.* 2014). A second exception may involve the *KCNK5* gene whose mRNA expression level inversely correlates with aldosterone synthesis and microRNA expression in a subcohort of 32 confirmed APA patients of Italian origin (Rossi *et al.* 2006*a*; Lenzini *et al.* 2014). While the universality of these findings awaits their replication in multiethnic cohorts, the data in aggregate indicate that APA is a disorder with highly variable cellular and molecular phenotypes with diverse genetic underpinnings.

Despite the lack of consistent genotype–phenotype correlations (Boulkroun *et al.* 2015; Zennaro *et al.* 2015), the bulk of evidence supports the hypothesis that independent, mutational-based mechanisms can support a persistent elevation in $Ca^{2+}$ that drives the overproduction of aldosterone. Specifically, all of these mutations – directly or indirectly – are expected to raise intracellular $Ca^{2+}$ in ZG cells, either by an increase in $Ca^{2+}$ channel open probability ($Ca_V1.3$, $Ca_V3.2$), by a loss of PMCA3 $Ca^{2+}$ pump activity, or by a depolarization in cell membrane potential, an action predicted to reduce $Na^+$–$Ca^{2+}$ exchange activity and/or activate voltage-dependent $Ca^{2+}$ channels.

## Calcium sites of action and regulation of the $Ca^{2+}$ signal

In the ZG cell, $Ca^{2+}$ is *the* critical second messenger that regulates the production of aldosterone. The steroidogenic, intracellular $Ca^{2+}$ signal is generated by the opening of $Ca^{2+}$-selective, voltage-dependent channels found at the plasma membrane, or by the release of $Ca^{2+}$ from intracellular stores (Fakunding & Catt, 1980; Kojima *et al.* 1984; Barrett *et al.* 1989; Rasmussen *et al.* 1989). The subsequent increase in cytosolic $Ca^{2+}$ facilitates the delivery of cholesterol to the mitochondria (Cherradi *et al.* 1996) for its conversion to pregnenolone, a precursor in the aldosterone biosynthetic pathway. To *persistently* increase steroidogenesis, the $Ca^{2+}$ signal must also be transferred to the mitochondrial matrix (Lalevee *et al.* 2003) where it stimulates matrix dehydrogenases to generate NADH. NADH, in turn, is converted to NADPH, a cofactor that is required for two critical regulatory steps in the biosynthetic pathway: (1) the conversion of cholesterol to pregnenolone catalysed by CYP11A1, and (2) the conversion of deoxycorticosterone to aldosterone catalysed by CYP11B2 (Rossier *et al.* 1996; Wiederkehr *et al.* 2011). Thus, a $Ca^{2+}$ increase in both the cytosolic and the mitochondrial compartments of the ZG cell is required to increase the production of aldosterone that persists for minutes to hours (Spat & Hunyady, 2004).

## Voltage-gated calcium currents

Early electrophysiological recordings in rat, bovine and human ZG cells consistently identified two components of the $Ca^{2+}$ current that could be attributed to distinct $Ca^{2+}$ channel classes based on voltage-dependent gating properties, kinetics and pharmacology (Matsunaga *et al.* 1987; Cohen *et al.* 1988; Durroux *et al.* 1988; Payet *et al.* 1994). Typically, the more persistent current that was elicited by strong depolarization (from resting $V_m$ of $-85$ mV to $> -50$ mV ($Ca_V1.3$), or $> -30$ mV ($Ca_V1.2$)) (Xu & Lipscombe, 2001) and inhibited by dihydropyridines characterized the high voltage-activated component (HVA). By contrast, the rapid, transient current that was evoked by weak depolarization (from resting $V_m$ to $> -65$ mV) and inhibited by $Ni^{2+}$ characterized the low voltage-activated component (LVA) (Tsien *et al.* 1988). Later, the molecular correlates of these currents were identified as HVA, L-type (i.e. $Ca_V1.3$ and $Ca_V1.2$) and LVA, T-type (i.e. $Ca_V3.2$ and $Ca_V3.1$) channels. The relative mRNA abundance of L- and T-type channel isoforms in adrenal ZG varies among studies and species (Lesouhaitier *et al.* 2001; Schrier *et al.* 2001; Rossier *et al.* 2003) although their biophysical properties remain similar. Additionally, functional N-type channels ($Ca_V2.0$) have been recorded in isolated rat ZG cells (Durroux *et al.* 1988). Thus, the repertoire of $Ca^{2+}$ channels found in ZG cells is rich.

More recently, the expression profiles for voltage-gated $Ca^{2+}$ channels in normal and pathological human adrenocortical tissue were compared (Scholl *et al.* 2013; Felizola *et al.* 2014). mRNA for all three $Ca^{2+}$ channel classes was detected in human aldosterone-producing adenomas, with greater relative abundance of $Ca_V1.3$ and $Ca_V3.2$

mRNA than $Ca_V1.2$ and $Ca_V2.2$ mRNA (Felizola *et al.* 2014). Surprisingly, the protein expression of $Ca_V1.3$ and $Ca_V3.2$ channels is consistent regardless of tissue health, as anti-$Ca_V1.3$ or anti-$Ca_V3.2$ immunoreactivity is equivalent in APA, IHA and normal adrenocortical tissue samples. However, whether all detected protein is functional remains a critical unanswered question.

## Calcium homeostasis: lessons learned from isolated cells

From the late 1970s to early 1990s, methods to dissociate and isolate ZG cells from bovine and rat adrenal tissues led to tractable preparations suitable for measuring ZG cell $Ca^{2+}/K^+$ currents, intracellular $Ca^{2+}$ signals and aldosterone production. Numerous studies document that the major regulators of aldosterone production, angiotensin II and extracellular $K^+$, raise intracellular $Ca^{2+}$ in ZG cells (Capponi *et al.* 1984, 1987; Connor *et al.* 1987; Kramer, 1988; Johnson *et al.* 1989; Pratt *et al.* 1989; Rossig *et al.* 1996) and that changes in cell $Ca^{2+}$ are commensurate with a striking sustained depolarization of the ZG cell. Angiotensin II elicits ZG cell depolarization by inhibiting hyperpolarizing conductances, predominantly those mediated by leak and voltage-gated $K^+$ channels (Quinn *et al.* 1987*b*; Brauneis *et al.* 1991; Lotshaw, 1997*a*; Guagliardo *et al.* 2012), whereas elevated extracellular $K^+$ induces a shift in the $K^+$ equilibrium potential across the highly $K^+$-conductive ZG plasma membrane (Quinn *et al.* 1987*a*,*b*; Lotshaw, 1997*b*). As depolarization recruits the activation of voltage-gated $Ca^{2+}$ channels, these early findings suggested the possibility that voltage-gated $Ca^{2+}$ entry is an important contributor to ZG cell activation and aldosterone production.

With the subsequent development of pharmacological agents to reduce the open-state probability of selective $Ca^{2+}$ channel classes, as well as hormonal and molecular approaches to alter $Ca^{2+}$ channel gating mechanisms, the relative contribution of T-type and L-type $Ca^{2+}$ currents to the regulation of aldosterone production was confirmed. Given the extremely hyperpolarized resting membrane potential of isolated ZG cells ($\sim -85$ mV), the relatively modest ~5–20 mV depolarizing shifts in voltage evoked by physiological concentrations of angiotensin ll or $K^+$, coupled with robust T-type channel currents recorded in all preparations, it is not surprising that T-type and not L-type $Ca^{2+}$ current amplitude (Barrett *et al.* 1995; Rossier *et al.* 1996; Lotshaw, 2001) strongly correlated with aldosterone output in these studies (data from 9 previous studies compiled and reviewed by Rossier (2006)). Thus, voltage-gated $Ca^{2+}$ channels have assumed a privileged role in ZG cell $Ca^{2+}$ homeostasis.

Framed by the aforementioned and other studies, the ZG cell came to be regarded as electrically quiescent, operating over a narrow, relatively hyperpolarized voltage range in which the open probability of T-type $Ca^{2+}$ channels is low, but also in which the steady-state inactivation of the channel is incomplete. The latter, it was argued, promoted a small-yet-discernible $Ca^{2+}$ conductance into the ZG cell via a T channel 'window current' that was functionally sufficient to sustain the production of aldosterone (Cohen *et al.* 1988; Lotshaw, 2001; Wolfe *et al.* 2002). However, at odds with this description of an electrically silent ZG cell were several findings: (1) angiotensin II elicited oscillatory changes in cytosolic free $Ca^{2+}$ in a small percentage of isolated cells that persisted for several minutes before transitioning to a tonic elevation (Johnson *et al.* 1989); (2) oscillatory $[Ca^{2+}]_i$ responses preferentially evoked by low concentrations of angiotensin II (Quinn *et al.* 1988) depended only on voltage-gated $Ca^{2+}$ entry and not $IP_3$-induced $Ca^{2+}$ release (Rossig *et al.* 1996); (3) spontaneous $Ca^{2+}$-dependent voltage spike potentials were recorded in ZG cells retained within cat adrenal slices (Natke & Kabela, 1979); and (4) spike potentials in isolated ZG cells could be evoked by depolarizing current injection or by pharmacological blockade of $K^+$ currents (Quinn *et al.* 1987). More recently, our laboratory extended these findings and discovered that ZG cells are indeed electrically excitable when their cellular connectivity is preserved within a tissue slice (Hu *et al.* 2012). Specifically, ZG cells organized within cortical rosette structures spontaneously generate periodic (~0.5 Hz), large depolarizing amplitude changes ($\Delta$ +75 mV from −85 mV) in membrane potential ($V_m$ oscillations) that are modulated in frequency by angiotensin II and extracellular $K^+$. Collectively, these observations demonstrate that $Ca^{2+}$ levels within ZG cells are highly dynamic and, therefore, motivated a further evaluation of the excitability of the ZG cell.

## Pacemakers in the zona glomerulosa

Many excitable cells, ranging from those found in invertebrate (Lewis, 1988; Lamb & Calabrese, 2012; Marder *et al.* 2015) to mammalian (Steriade *et al.* 1993; Marcantoni *et al.* 2010; Vandael *et al.* 2010) systems, have the capacity to generate electrical oscillations, even when isolated. In many cases, these intrinsic oscillations are driven by a small ensemble of distinct ion channels that operate in concert to generate periodic changes in voltage. These so-called *pacemaker* channel conductances often rely on voltage-gated $Ca^{2+}$ channels to produce the large, rising phase that characterizes the depolarizing component of the voltage oscillation. For example, a well-characterized oscillator reliant upon T-type $Ca^{2+}$ channels is found in thalamocortical neurons of the thalamus, in which an interplay between T-type $Ca^{2+}$ channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels drives voltage

oscillations likely to be important for memory consolidation during sleep (Gais *et al.* 2002; Eschenko *et al.* 2006; Fogel *et al.* 2011). A second, well-characterized oscillator reliant upon voltage-gated $Ca^{2+}$ channels is in the catecholamine secreting adrenal chromaffin cell in which there is an interplay between $Ca_V1.3$ channels and $Ca^{2+}$-activated $K^+$ channels (BK, SK). In this cell, the relatively low threshold for activation of $Ca_V1.3$ currents at interspike potentials ($\sim -50$ mV) drives depolarization and $Ca^{2+}$ entry which is sufficient to activate BK channels within restricted $Ca^{2+}$ domains and highly $Ca^{2+}$-sensitive SK channels at more distal sites. Once active these $K^+$ channels hyperpolarize the membrane, remove $Ca_V1.3$ channel inactivation and enable the initiation of a new oscillatory cycle (Marcantoni *et al.* 2010; Vandael *et al.* 2010; Vandael & Carbone, 2015).

The recurrent observation that biological pacemakers often recruit voltage-gated $Ca^{2+}$ channels to produce oscillations motivated our laboratory to identify ion channels that endow the ZG cell with the capacity to operate as a pacemaker. While such identification is incomplete, we have learned that targeting $Na_V1.x$ channels with TTX or $Ca_V1.x$ channels with nifedipine fails to alter either the amplitude or the frequency of ZG $V_m$ oscillations. In contrast, low concentrations of $Ni^{2+}$ that selectively target $Ca_V3.x$ channels halt $V_m$ oscillations, indicating an important contribution of these low voltage-activated $Ca^{2+}$ channels to ZG pacemaking (Hu *et al.* 2012). Considering that $Ca_V3.2$ is the primary $Ca_V3.x$ member expressed in the ZG layer, we hypothesize that activation of $Ca_V3.2$ channels underlies the rapidly depolarizing phase of the ZG cell oscillation. The identification of the ion channel(s) that contribute to the falling phase remains less well delineated. However, silencing $V_m$ oscillations with $Ni^{2+}$ does not cause a hyperpolarization as would be predicted from block of T-type $Ca^{2+}$ current, but rather causes a 20 mV depolarization from baseline (to $\sim -60$ mV), suggestive of a functional link between $Ca_V3.2$ and a $Ca^{2+}$-activated $K^+$ current(s). In this model, $Ca^{2+}$ entry through $Ca_V3.2$ channels both depolarizes the ZG cell and recruits $Ca^{2+}$-activated $K^+$ channels which serve to hyperpolarize the cell; thus, the $Ni^{2+}$-evoked depolarization likely reflects the closure of the latter. It is notable that the peak amplitude of $Ca_V3.2$ pacemaker current is during the repolarization phase of the oscillatory cycle; this temporal dependence would allow $Ca_V3.2$ current to activate $Ca^{2+}$-activated $K^+$ channels to complete the oscillatory cycle. Together these data provide evidence, albeit indirect, that a $Ca^{2+}$-activated $K^+$ conductance may participate in the falling phase (hyperpolarization phase) of the ZG cell oscillation (Fig. 1).

The aforementioned ZG pacemaker model is developed from observations amassed from normal, wild-type mice. In adapting these findings to human ZG cells, or to ZG cell behaviour during pathological conditions, it is important to consider the potential contribution of L-type $Ca^{2+}$ channels ($Ca_V1.3$), the mRNA of which is also found in normal human adrenals, aldosterone-producing adenomas (Azizan *et al.* 2013; Scholl *et al.* 2013; Fernandes-Rosa *et al.* 2014) and recently in aldosterone-producing clusters (Nishimoto *et al.* 2015) in addition to that for $Ca_V3.2$. Two salient features of $Ca_V1.3$ channels are worth noting when considering their contribution to ZG function. First, the voltage range over which $Ca_V1.3$ channels carry $Ca^{2+}$ current is $\sim$25 mV more hyperpolarized than prototypical L-type $Ca^{2+}$ channels, potentially enabling them to be active within the voltage range of the ZG cell oscillation ($-85$ to $-10$ mV). Although $Ca_V1.3$ channels would not be active at rest ($-85$ mV), they would be recruited in the oscillatory cycle following activation of lower threshold depolarizing conductances, including $Ca_V3.2$ channels. However, because $Ca_V1.3$ channels undergo rapid $Ca^{2+}$-dependent inactivation (CDI) (Xu & Lipscombe, 2001), it remains unclear to what extent these channels contribute to ZG pacemaker function or aldosterone production under non-pathological conditions. Indeed, it is worth noting that in animal species that express T-type and L-type $Ca^{2+}$ channels (rat, bovine), T-type current amplitude but not L-type current correlates with aldosterone production (Barrett *et al.* 1995; Rossier *et al.* 1996*b*; Lotshaw, 2001; Rossier, 2006).

By contrast, it is likely that the contribution of mutant $Ca_V1.3$ channels to exaggerated aldosterone production is substantial. As discussed above, many gain-of-function mutations in the *CACNA1D* gene encoding $Ca_V1.3$ channels are observed in aldosterone-producing adenomas (Azizan *et al.* 2013; Scholl *et al.* 2013; Fernandes-Rosa *et al.* 2014) and aldosterone producing clusters (Nishimoto *et al.* 2015). As many of these mutations shift $Ca_V1.3$ gating properties to substantially more hyperpolarized membrane voltages, the reliance of $Ca_v1.3$ channel activation on lower threshold conductances would be mitigated, thereby allowing channels to open at rest and also to conduct $Ca^{2+}$ under a greater inward driving force. In principle $Ca^{2+}$ entry could also be augmented by disrupting CDI (Xu & Lipscombe, 2001). Based on structure–function analysis of $Ca_V1.3$ channels, mutations that alter activation gating but spare voltage-dependent inactivation (VDI) would be most likely to alter CDI (Tadross *et al.* 2010). However, to date most mutations in APAs have not been experimentally tested for disrupted CDI and in the one study that has, CDI remained unperturbed (Scholl *et al.* 2013). Nevertheless, the modulation of this channel gating property of $Ca_V1.3$ could provide an additional mechanism for regulating $Ca^{2+}$ entry and cellular excitability by altering the rate of spontaneous and evoked oscillations independent of changes in the

voltage gating properties of $Ca_V1.3$ channels (see Fig. 1; Scharinger *et al.* 2015).

## Potential relevance of ZG oscillations

If we accept the new place of the ZG cell among pacemakers, what then is the physiological relevance of ZG voltage oscillations? Do ZG cell voltage oscillations simply exist to recruit a range of voltage-dependent $Ca^{2+}$ conductances that collectively ensure a significant rise in cytosolic $Ca^{2+}$ such that aldosterone is produced? In such a context, ZG $Ca^{2+}$ oscillations would be epiphenomenal, i.e. a large, non-periodic increase in cytosolic $Ca^{2+}$ could achieve the same effect. Or do periodic oscillations provide a unique opportunity for $Ca^{2+}$ channels with divergent biophysical properties to subserve singular functions in regulating ZG cell activity and aldosterone production? Support for such a possibility comes directly from ZG studies and also from many more summarized and formalized by McCobb and Beam (1991).

First, as determined by oscillatory voltage-clamp experiments conducted in ZG cells, ZG $V_m$ oscillations are sufficient in magnitude and correct in frequency to drive repetitive, large-amplitude $Ca_V3.2$-mediated currents known as low threshold calcium spikes. Thus, $V_m$ oscillations in ZG cells provide a mechanism for rapidly inactivating $Ca_V3.2$ channels to generate long-lasting, periodic $Ca^{2+}$ signals in ZG cells that would be much larger than those associated with T-channel window currents reliant upon incomplete channel inactivation (Hu *et al.* 2012). Second, because T-type $Ca^{2+}$ channels have the lowest threshold for activation and have a relatively slow rate of closure (deactivation), $Ca^{2+}$ entry through these channels is driven by more negative driving forces. Thus, $Ca^{2+}$ entry can be quite large and is favoured in response to a rapidly repolarizing oscillatory wave-form (McCobb & Beam, 1991), such as recorded in ZG cells. Third, because L-type $Ca^{2+}$ channels have a more depolarized inactivation threshold and slower inactivation kinetics, entry through L-type $Ca^{2+}$ channels would be favoured in response to oscillatory waveforms that decay/repolarize slowly and have extended durations. Thus, as has been elegantly demonstrated and formalized by McCobb and colleagues, because of the high sensitivity of L-type $Ca^{2+}$ channels to the width of the oscillatory waveform, L-type $Ca^{2+}$ channels would provide the ZG cell with a mechanism to report activity changes in $K^+$ channels that control the rate of repolarization. By contrast, given the relative insensitivity of T-type $Ca^{2+}$ channels to oscillatory waveform width (McCobb & Beam, 1991), $Ca^{2+}$ entry through T-type channels would better report ZG oscillation frequency. Thus, although each channel type can convert an electrical signal into a chemical $Ca^{2+}$ response, when presented with an oscillatory electrical signal they do so advantaged by their biophysical properties. Thus, ZG $V_m$ oscillations may allow the cell to functionally compartmentalize the contribution of distinct voltage-gated $Ca^{2+}$ channels.

Finally, it is important to consider how ZG cells might process a periodic $Ca^{2+}$ signal and how such a signal could control the rate of aldosterone production. In this regard the biochemical and computational studies of $Ca^{2+}$/calmodulin-dependent protein kinase (CaM kinase II) are noteworthy. In this multimeric enzyme complex, individual subunits of CaMKII are activated by high threshold periodic pulses of $Ca^{2+}$. After the threshold for kinase activation is attained, some subunits are trans-phosphorylated at $Thr^{286}$ to generate a persistent, subthreshold kinase activity that is autonomous of the

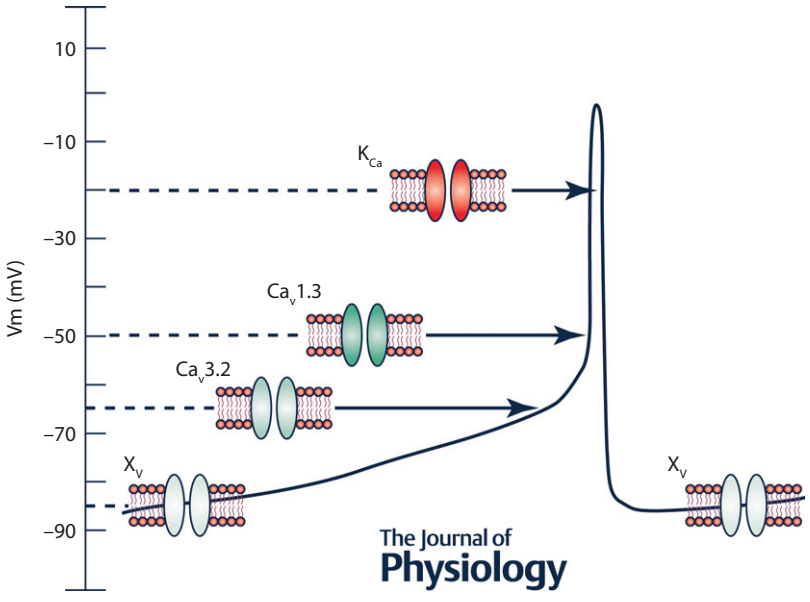


**Figure 1. A working model of the ZG pacemaker**
At the start of the oscillatory cycle ($V_m = -85$ mV), depolarizing conductance(s) ($X_v$) elicit the opening of low voltage-activated $Ca_v3.2$ channels which further depolarize $V_m$ and enable the opening of high voltage-activated $Ca_v1.3$ channels. $Ca^{2+}$ influx and a reduced $V_m$ recruits the activity of $Ca^{2+}$-dependent $K^+$ channels that return $V_m$ to −85 mV to begin another oscillatory cycle.

$Ca^{2+}$ signal (Hanson *et al.* 1989). This positive feedback loop augments responses to low frequency stimuli and allows CaMKII to act as a decoder of the frequency of oscillatory $Ca^{2+}$ signals (De Koninck & Schulman, 1998). In a similar manner, it is formally possible that periodic $Ca^{2+}$ signals within ZG cells are more potent drivers of aldosterone production than static $Ca^{2+}$ signals, because in ZG cells CaMKII activation induces a hyperpolarizing shift in the $V_{1/2}$ of activation of T-type channels (Welsby *et al.* 2003; Yao *et al.* 2006). Such an increase in T-current amplitude could thus provide a greater depolarizing current for L-type channel recruitment further increasing extracellular $Ca^{2+}$ entry. This possibility awaits future examination.

## Final remarks

Here, we provide a brief review of recent mutational analyses performed on human adrenal tissues associated with excessive aldosterone production, and address how the newly defined ZG pacemaker cell, by driving the cell to membrane potentials that support channel activity, may unmask these mutations, many of which are associated with voltage-dependent ion channels. In attempting to address the latter, we are confronted with many uncertainties. Clearly, to completely understand how a mutated ion channel contributes to excessive steroidogenic signalling, we must first better define the collection of ionic conductances that govern ZG cell excitability in the normal, healthy state. And yet, our current knowledge of the ionic conductances that drive ZG pacemaker activity is primarily limited to how the low threshold, T-type $Ca^{2+}$ channel ($Ca_V3.2$) drives ZG membrane potential to depolarized levels. To this end, we must also define all currents that mediate the rapid repolarization phase of the ZG pacemaker oscillation. Only when all the relevant channels comprising the ZG pacemaker are identified and the pacemaker regulation of aldosterone production is known, will we be able to fully understand by what means specific mutations support greater aldosterone production. Clearly, the future remains an exciting time for ion channel physiology within the realm of adrenal function.

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## Additional information

### Competing interests

The authors have no competing interests.

### Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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