Subtle mutation, far-reaching effects

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In the 1980s, medical research set its focus more and more on molecular aspects of diseases, and it was not long until an individual mutation in a single protein species was found to cause the complete phenotype of a disease. Unsurprisingly, ion channels were among those culprits. Significant public attention was aroused when mutations in the (then appropriately named) cystic fibrosis transmembrane conductance regulator (CFTR)—a chloride channel—were identified as initiators of cystic fibrosis (Riordan et al., 1989). Within years, an entire zoo of channel-dependent diseases was discovered, and the term "channelopathy" was introduced to describe them. It is perhaps not a surprise, then, to find that mutations in ion pumps also cause disease. But because cells get along with about a dozen of ion pumps, in contrast to hundreds of channels, the number of pump-induced diseases is rather limited and the notation "pumpopathy" will probably not be needed to embrace them. In this issue of *The Journal of General Physiology*, Meyer and colleagues investigate one such pump-induced disease and define the molecular mechanism that underlies it.

The Na^+,K^+-ATPase—a member of the P-type ATPase family—is an essential ion transporter in virtually all animal cells. This pump exists in several isoforms to handle the specific metabolic needs of each cell type. Numerous clinical conditions have been correlated with modified Na^+,K^+-ATPase activity for decades and are mainly caused by alteration of endogenous or xenobiotic factors (Rose and Valdes, 1994). In 2004, however, a specific mutation in the α2 isoform of the Na^+,K^+-ATPase was found to cause familial hemiplegic migraine (Swoboda et al., 2004), and in the years that followed, further mutations were identified to cause various forms of migraine (Friedrich et al., 2016). More recently, it has been found that mutations in the neuron-specific Na^+,K^+-ATPase α3 subunit are linked to rapid-onset dystonia Parkinsonism (Shrivastava et al., 2015) and that the α3 subunit may play also a role in the neurodegeneration of Alzheimer patients (Ohnishi et al., 2015).

There is another disease, primary aldosteronism, which causes secondary hypertension by overproduction of aldosterone, that is provoked—among other reasons—by single mutations of the α1 isoform of the Na^+,K^+-ATPase in adenomas within the zona glomerulosa of the adrenal cortex. In recent years, mutations of five residues in the α1 subunit have been found to cause overproduction of aldosterone: G99R, L104R, delF100-L104, V332G, and EETA963S (Azizan et al., 2013; Beuschlein et al., 2013; Williams et al., 2014).

The chain of events that lead to aldosterone production in the adrenal cortex begins with transient membrane depolarizations initiated by angiotensin II or hyperkalemia followed by an increase in intracellular Ca^{2+} concentration. As a second messenger, intracellular Ca^{2+} triggers increased transcription of the CYP11B2 gene that encodes aldosterone synthase, and so leads to an enhancement of aldosterone production (Fig. 1 A). In the case of hyperaldosteronism, as it is found in aldosterone-producing adenomas, constitutive aldosterone production is detected without physiological triggers.

Assuming that the intracellular chain of events that begins with increased Ca^{2+} is unchanged, the pathological culprit has to be part of the mechanism that leads to this boost of Ca^{2+}. There are several possible mechanisms that could produce such an enhanced Ca^{2+} concentration: Mutations of Ca or K channels, the plasma membrane Ca-ATPase (PMCA), or the Na^+,K^+-ATPase. Modified Ca channels or gradually malfunctioning Ca-ATPases could directly lead to enhanced Ca^{2+} concentrations. Altered K channels could cause loss of cytoplasmic K^+, which would depolarize the resting potential and thus provoke increased opening of voltage-dependent Ca channels. However, when mutations of the Na^+,K^+-ATPase are the initiators, it must be an indirect way that triggers the gain of intracellular Ca^{2+}. But even if one restricts the investigation to the Na^+,K^+-ATPase, it turns out that the underlying molecular mechanism is not just one clear-cut process, as it is revealed in the paper of Meyer et al. (2017).

A couple of years ago, studies of the first three mutations causing primary aldosteronism, L104R, delF100-L104, V332G, were performed with cells from adenoma primary cultures and COS cells transfected with cDNA encoding wild-type Na^+,K^+-ATPase or one of these mutations. The crucial finding was that these mu-

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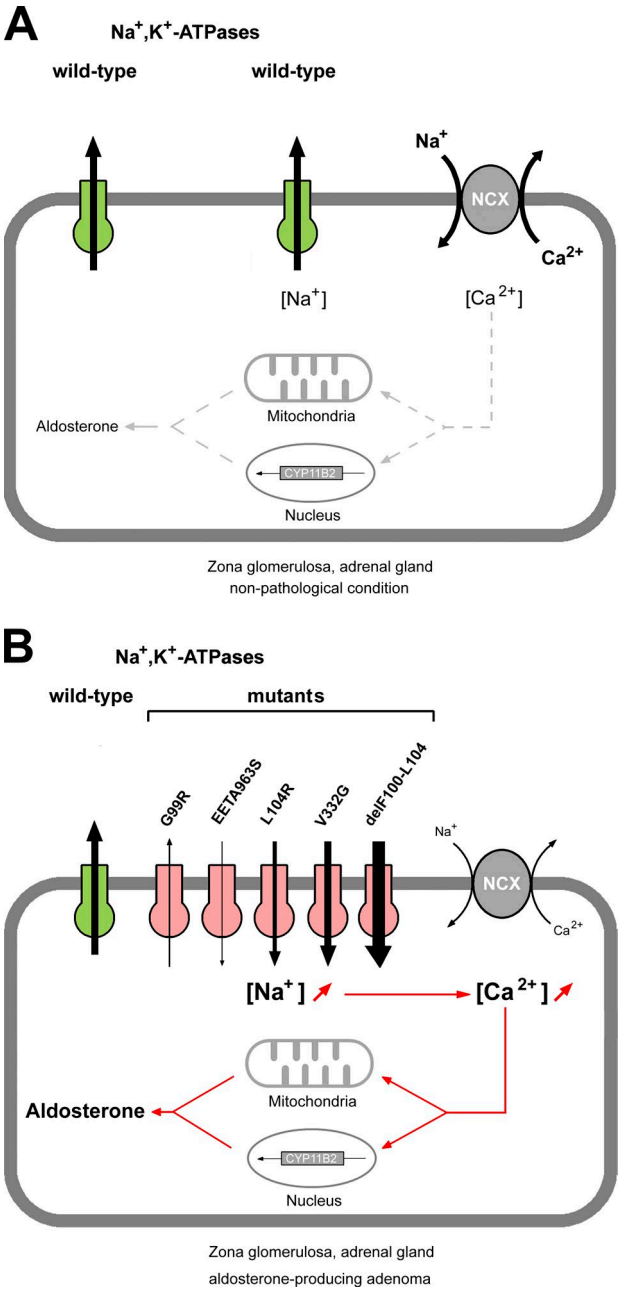

Figure 1. **Aldosterone production in cells of the adrenal gland.** (A) Under nonpathological conditions, the intracellular Na^+ concentration is maintained low by an adequate Na^+ extrusion capacity provided by wild-type Na^+,K^+-ATPases. This allows for proper calcium extrusion by the Na/Ca exchanger (NCX). Physiological depolarizing fluctuations of the membrane potential lead to transient openings of Ca channels, and a temporarily enhanced Ca^{2+} concentration provokes aldosterone production satisfying the aldosterone requirements of the organism. (B) In specific aldosterone-producing adenomas, expression of (monoallelic) mutant Na/K pumps reduces the Na^+ extrusion performance to ~50% and adds inward leaking Na^+ currents (except in the case of the G99R mutant). The arrows through each "pump" insinuate the relative sizes and directions of the ouabain-sensitive currents. The pump malfunctions elevate the intracellular Na^+ concentration, leading to reduced Ca^{2+} extrusion by NCX and, consequently, to increased intracellular Ca^{2+} concentrations. Elevated intracellular Ca^{2+} (as secondary messenger) increases StAR-dependent cholesterol transport in the mitochondria (the early step of aldosterone synthesis) and transcription of CYP11B2 (i.e., aldosterone synthase; the late step of synthesis), leading to a sustained production of aldosterone. Not included in both schemes are the numerous secondary-active transporters that use the inward directed electrochemical potential gradient of Na^+ to transport other substrates needed in the cell metabolism on expense of further Na^+ inward currents that are compensated entirely by the wild-type Na^+,K^+-ATPase under nonpathological conditions. (The schemes are derived from a slide presented by D. Meyer.)

tations caused a significant depolarization of the resting membrane potential in the order of 20 mV (Beuschlein et al., 2013). Shortly afterward, a fourth mutation, EETA963S, was published in a paper that presented results from electrophysiological experiments with oocytes in which Na^+,K^+-ATPases of all four mutations were expressed separately (Azizan et al., 2013). They reported that, under near-physiological conditions, all four mutations resulted in Na^+,K^+-ATPases that have exhibited "distinct inward, ouabain-sensitive currents in Na^+." Normally, the Na^+,K^+-ATPase produces a net outward current of positive charges as the result of the transport stoichiometry of 3 Na^+ out of and 2 K^+ ions into the cell.

Those steady-state inward currents through mutant Na^+,K^+-ATPases are undesired leak currents that may lead to a depolarization of the membrane potential, consistent with the observations reported on the adenoma primary culture cells. This additional contribution of the Na^+,K^+-ATPase to transport processes through the membrane has been termed "gain-of-function." When in aldosterone-producing adenomas the G99R mutation was found to also induce primary aldosteronism, it could be established that an overexpression of this mutant in human embryonic kidney cells led to a depolarization of the resting potential of the membrane (Williams et al., 2014). Because this effect was observed even in the absence of external Na^+, it could not be caused by a leak Na^+ inward current, but may possibly be the consequence of a reduced functional activity of the pump.

To shed light on the pathways of the ions leaking through the mutated Na^+,K^+-ATPase and to find out by which mechanism they may cause the increased Ca^{2+}concentration that triggers the autonomous hypersecretion of aldosterone, Meyer et al. (2017) set up an elaborate experimental approach. In their survey, the five mutants of the human Na^+,K^+-ATPase were expressed in *Xenopus* oocytes, and the pump-induced electric currents were measured by the two-electrode voltage clamp technique. In the same oocytes, the number of contributing pumps was determined by their inhibition with ^{3}H-labeled ouabain and the detection of the amount of bound radioactivity. From this measure, together with the corresponding electric current, they

were able to determine the effective turnover number of the pumps. In additional experiments, the uptake of radioactive $^{22}Na^+$ and $^{86}Rb^+$ (a well-established congener of K^+) was measured to follow the actual ion fluxes. Double mutants, in which the D933N mutation was introduced alongside the aldosteronism-inducing mutation, allowed the authors to determine whether the leaking ions passed through ion-binding site III or II. A wealth of control experiments enabled the authors to present a "waterproof" argument in support of their mechanistic proposal.

Because the "gain-of-function" that was proposed for those mutations resulted in Na^+ influx, it was interesting to elucidate the pathway for these ions. The physiological concept of ion transport through the Na^+,K^+-ATPase is that of a "gated ion channel" (Apell, 2017). In this model, the ion-binding sites, which are almost in the middle of the membrane domain of the ion pump, are alternatingly accessible through a narrow access channel from one side of the membrane via an open gate, whereas the other access channel is blocked by a closed gate. The state of the gates is strictly controlled. In the E1 conformation, the gate is opened that allows ion exchange with the cytoplasm. In the E2P state, the other gate is unlocked to permit the exchange with the extracellular phase. During the transition between both conformations, an intermediate state, the so-called occluded state, occurs in which both gates are closed. This way, a short circuit is prevented that would cause a counterproductive passive ion flux. When the location of the scrutinized mutated amino acids was checked by comparison with the crystal structure of the protein, it turned out that all mutations are close to the ion-binding sites II and III, which are assigned to the second and third Na^+ ion bound. These mutations may cause a malfunction in one of the aforementioned gates and thus promote the observed leak currents.

To answer the question of whether the leaking Na^+ ions pass through binding site II or III, the authors constructed double mutants in which, in addition to L104R, delF100-L104, V332G, or EETA963S, the mutation D933N was introduced. The latter is known to block site III and prohibit active and leak ion transport through this site (Vedovato and Gadsby, 2014). It turned out that in one of the mutants, EETA963S/D933N, the leak current was indeed reduced to a mere 10% of the current observed for the EETA963S-only mutation. This finding indicates that the leak current in the EETA963S mutant flows through a pathway different from that of the three other mutants. This result is consistent with the proposal that, based on the location of the four mutations in the crystal structure, the leak current pathway in the EETA963S mutant includes site III, whereas in the others it includes site II (Kopec et al., 2014).

When Meyer et al. (2017) studied the EETA963S mutant in detail, they found that, at physiological salt concentrations (i.e., 125 mM Na^+_o and 4.5 mM K^+_o), the leak inward current disappeared completely at a membrane voltage of −50 mV. Bearing in mind that cells exhibit monoallelic mutations in aldosterone-producing adenomas (Beuschlein et al., 2013), so that only 50% of the expressed Na^+,K^+-ATPases are mutated, it is reasonable to conclude the following: If half of the pumps generate the normal outward current and these mutated pumps do not generate significant inward leak currents, it is unlikely that such a condition causes a membrane depolarization large enough to trigger an autonomous hypersecretion of aldosterone. Therefore, they performed a systematic analysis of the inward leak currents of all four mutants and compared them with the outward current of the (not mutated) wild-type pump under identical conditions. To obtain a parameter that allows a direct comparison of the properties of single pumps, they determined the "turnover rate" of each pump species, i.e., the number (and direction) of elementary charges transported per second and pump molecule. This approach was implemented by measuring the ouabain-dependent current through the membrane of an oocyte and determining the amount of ouabain bound to the same oocyte by using ^3H-labeled compound. With knowledge of the stoichiometry of one ouabain bound per pump and some simple math, they calculated the desired rate with less than ∼10% error.

The results were surprising: Even under optimal "leak" conditions for the mutants (i.e., high Na^+_o and 0 K^+_o), only one species, delF100-L104, had a high turnover rate of 16 times that of the wild type. In the case of V332G, the rate was only twice that of the wild type, and in the case of the remaining two mutants, it was less than that of the wild type. At least for the latter two, L104R and EETA963S, the proposal that their operation is able to produce a considerable depolarization of the membrane potential has to be abandoned when in competition with properly working wild-type pumps in the same membrane.

The next building block of the study was the characterization of the electrophysiological properties of the most recently found mutant containing G99R. This amino acid resides in TM1 and is located close to binding site II. This mutant also causes hyperaldosteronism in adenoma cells. But when expressed in oocytes, its physiological behavior resembled more wild-type Na^+,K^+-ATPase than one of the four other mutants: It lacked completely inward currents, no ouabain dependent uptake of ^{22}Na was observed, and it generated a minor outward current under physiological electrolyte conditions with a turnover rate of 75% of the wild type. Only the ion-binding affinities for Na^+ and K^+ were reduced in both conformations of the pump when compared with the wild-type Na^+,K^+-ATPase. Meyer et al. (2017) summarized that the observed changes in the

G99R mutant "induce a major loss-of-function under physiological conditions."

This term "loss-of-function" provided the keyword for an alternative mechanistic proposal to explain hyperaldosteronism in adenoma cells. The evidence they present in their paper is as follows: (a) extracellular K^+, which is available under physiological conditions, reduces the amplitude of the "leak currents" through all four "leaky" mutants; (b) with exception of the delF100-L104 mutant, the inward currents of the other mutants are not high enough to override the normal outward currents of the intact wild-type pumps, which are present in the membrane in comparable amount. Thus, leak currents as "gained function" of the mutants will not be sufficient to provoke a considerable depolarization of the membrane's resting potential in four of the five mutants.

An obvious question is, of course, whether the loss of the outward current of Na^+ in four of the mutants, and a significantly reduced Na^+ current in the case of G99R, are sufficient to cause hyperaldosteronism. Because of haploinsufficiency, the pump capacity of the adenoma cells is reduced to 50%. In principle, a loss of the Na^+,K^+-ATPase activity induced by addition of ouabain has been shown to increase the aldosterone production in zona glomerulosa cells of rats (Yingst et al., 1999). In addition, it is known that inhibition of the Na^+,K^+-ATPase has only a minor effect on the resting potential in the order of a few millivolts. Such a small depolarization is not sufficient to cause an opening of voltage-gated Ca channels, the process which starts the chain reaction that culminates in aldosterone production under physiological conditions. What has to be expected, however, is an increase in cytoplasmic Na^+ concentration in the long run, and in the Na^+-leaking mutants anyway, but also in the G99R mutant with its considerably reduced Na^+ extrusion capacity.

These arguments, together with several additional points presented in the contribution of Meyer et al. (2017), encouraged the authors to present an alternative proposal (Fig. 1 B). Mutant Na^+,K^+-ATPases all have commonality that leads to an increase in cytoplasmic Na^+ concentration caused by reduced Na^+-pumping activity of each mutant and additionally by inward Na^+ leak currents of variable degree in the case of L104R, delF100-L104, V332G, and EETA963S. The turnover rates of the mutants (20–500 s^{-1}) indicate, however, that these leaks are not comparable with ion diffusion through a typical ion channel (with $>10^7$ s^{-1}) but more like a "disrupted ion transport" probably by destabilization of the occluded state. The increased cytoplasmic Na^+ concentration reduces the electrochemical potential gradient for Na^+ across the cell membrane, which is the driving force for Ca^{2+} extrusion by the Na/Ca exchanger. This secondary active transporter is a key player in Ca^{2+} export from cells in the zona glomerulosa (Kojima and Ogata, 1989). The functional interaction between the Na^+,K^+-ATPase and the Na/Ca exchanger leads to an increased steady-state Ca^{2+} concentration in the cytoplasm, and the continuous presence of this second messenger affects, according to the regular mechanism, the enduring production and secretion of aldosterone (Fig. 1 B).

Based on the results discussed, this proposal holds for all five mutants. The previous ("gain-of-function") proposal is based on a membrane depolarization of the order of 20 mV detected in aldosterone-producing adenoma cells. Such a depolarization would open voltage-dependent Ca channels and thus lead to aldosterone production by the usual route. Meyer et al. (2017) provide convincing evidence that an appropriate membrane depolarization in adenoma cells may be caused by the delF100-L104 mutant, potentially by the V332G mutant, but implausibly by the three other mutants.

This story is, however, not a contest between two proposals (and by no means is this intended), but a stimulating contribution that leaves the satisfying impression that this is additional insight that helps us to understand molecular details of a disease.

The pleasure of reading this paper was that, when following the authors through their presentation and—as is common to me in this process—asking as a critical reader at a certain point "But what if …", a satisfying answer was provided in the next (or one of the next) paragraph(s). In their systematic approach, no obvious objection or critical thought has been missed, resulting in a convincing piece of insight into a complex molecular mechanisms that eventually leads to the "phenotype" of primary aldosteronism caused by mutations of the Na^+,K^+-ATPase. And this makes it worth reading.

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