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Aldosterone regulation of T-type calcium channels☆

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Abstract

Voltage-operated calcium channels play a crucial role in signal transduction in many excitable and non-excitable cell types. While a rapid modulation of their activity by hormone-activated kinases and/or G proteins has been recognized for a long time, a sustained control of their expression level has been only recently demonstrated. In adrenal H295R cells, for example, aldosterone treatment selectively increased low threshold T-type calcium current density without affecting L-type currents. Antagonizing the mineralocorticoid receptor (MR) with spironolactone prevented aldosterone action on T-type currents. By RT-PCR, we detected in these cells the presence of two different isoforms of L-type channels, α_1C and α_1D, and one isoform of T channel, α_1H. A second T channel isoform (α_1G) was also observed under particular culture conditions. Quantification of the specific messenger RNA by real time RT-PCR allowed us to show a 40% increase of the α1H messenger levels upon aldosterone treatment (α_1G was insensitive), a response that was also completely prevented by spironolactone. Because T-type, but not L-type channel activity is linked to steroidogenesis, this modulation represents a positive, intracrine feed back mechanism exerted by aldosterone on its own production.

Aldosterone has been also implicated in the pathogenesis and progression of ventricular hypertrophy and heart failure independently of its action on arterial blood pressure. We have observed that, in rat neonatal cardiomyocytes, aldosterone increases (by two-fold) L-type calcium current amplitude in ventricular but not in atrial cells. No significant effect of aldosterone could be detected on T-type currents, that were much smaller than L-type currents in these cells. However, aldosterone exerted opposite effects on T channel isoform expression, increasing α_1H and decreasing α_1G. Although the functional role of T channels is still poorly defined in ventricular cardiomyocytes, an overexpression of α_1H could be partially responsible for the arrhythmias linked to hyperaldosteronism.

Finally, T channels also appear to be involved in the neuroendocrine differentiation of prostate epithelial cells, a poor prognosis in prostate cancer. We have shown that the only calcium channel expressed in the prostatic LNCaP cells is the α_1H isoform and that induction of cell differentiation with cAMP leads to a concomitant increase in both T-type current and α_1H mRNA. In spite of the presence of MR in these cells, aldosterone only modestly increased α_1H mRNA levels. A functional role for these channels was suggested by the observation that low nickel concentrations prevent neuritic process outgrowth.

In conclusion, it appears that T-type calcium channel expression vary in different patho-physiological conditions and that aldosterone, in several cell types, is able to modulate this expression.

Keywords: Aldosterone; T-type calcium channel; Adrenal steroidogenesis; Cardiomyocyte; Prostate cancer cell

1. Introduction

Calcium, as an ubiquitous intracellular messenger, plays a crucial role in many processes and its influx into the cells has to be precisely controlled. Because of their diversity, calcium channels, when simultaneously expressed in the same cell, could a priori appear as rather redundant for a function as simple as transporting calcium ions across the plasma membrane. In fact, it should be remembered that, due to the strict organization of the cell calcium signaling [1], specific roles can be attributed to each channel isoform. Indeed, the close environment of the channel, formed by different calcium-sensitive proteins or calcium-storing organelles, dictates the nature and the extend of calcium action at this precise location of the cell. For example, calcium entering a neuron across the pre-synaptic membrane, close to secretory granules, will induce neurotransmitter secretion, while calcium released next to a myofibril will be responsible of muscle contraction. In the case of steroidogenesis, mitochondrial calcium

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appears to control early steps of cholesterol conversion into steroids.

This diversity of calcium functions can, therefore, be maintained, even in the same cell, by a strict confinement of the signal, and explains the existence of specific calcium channelopathies associated with mutations affecting one single channel isoform. The specificity of the roles played by each channel probably prevents another isoform to compensate for the defective one.

A rapid modulation of the activity of voltage-operated calcium channels by various hormones and neuro-modulators has been recognized for a long time, and involves essentially heterotrimeric G proteins and protein kinase–phosphatase pathways [2]. More recently, a more sustained regulation of calcium influx, at the level of channel expression, has been also reported.

We have investigated the effect of aldosterone on the expression of low-threshold voltage-operated T-type calcium channels in three different cell models in which these channels play specific roles, either in physiological or pathological situations. In adrenal glomerulosa cells, T channels are specifically involved in the activation of aldosterone biosynthesis in response to extracellular potassium or angiotensin II [3,4], possibly because calcium entering the cell through these channels is selectively conveyed to the mitochondria, the site of early, rate limiting steps of cholesterol metabolism. In ventricular cardiomyocytes, T channels are poorly expressed during the adult life of most species, but could be involved, in case of re-expression, in various heart dysfunction, including cardiac arrhythmias that are often associated with heart failure and believed to be responsible for sudden death. Finally, in prostate cells, T channel expression has been recently shown to be linked to cancer cell neuroendocrine differentiation, a bad prognosis in tumorigenesis. We found that each of these tissues expresses the mineralocorticoid receptor (MR) but aldosterone appears to differentially affect the expression of their T channels and, therefore, some of the cell physiological function. A part of these results has been previously published elsewhere [5,6].

2. Materials and methods

2.1. Cell culture

Freshly isolated neonatal rat cardiomyocytes were maintained in primary culture, and the human adrenal H295R and prostate cancer LNCaP cell lines were grown, in their respective culture media, as previously described [5–7]. If not otherwise indicated, serum was removed from the medium 24 h before, and was absent during the treatment period with steroids and/or antagonists. Insulin, transferrin and selenium (ITS Plus) were obtained from Collaborative Biomedical Product (Bedford, MA), Ultroser SF from BioSepra (Villeneuve-la-Garenne, France), and fetal calf serum (FCS) and horse serum (HS) from Invitrogen (Basel, Switzerland).

2.2. Calcium channel activity

The activity of voltage-operated calcium channels has been assessed with the patch clamp technique in the whole cell configuration, using calcium or barium as charge carrier. Cells were voltage-clamped at −90 mV before to be depolarized at the indicated voltages.

2.3. Calcium channel expression

The expression of the various T- and L-type channel isoforms was quantitatively determined by measuring the respective messenger RNA levels by real time RT-PCR, using the Taqman or the SYBR Green techniques. Amplification was generally performed on extracted total RNA, reverse transcribed in the presence of random hexamers.

2.4. Statistics

Significance of the effect of treatments was assessed by the Student's *t*-test, a *P*-value <0.05 being considered as statistically significant.

3. Results and discussion

3.1. Aldosterone action on adrenal cortex T channels

Upon stimulation of adrenal glomerulosa cells by AngII, calcium is mobilized from intracellular stores and a capacitative influx is then activated. However, like extracellular potassium, AngII also induces a depolarization of the cell membrane and, therefore, activates voltage-operated calcium channels of both L- and T-types [8]. A part of this calcium is then transferred into the mitochondria, where it controls the limiting steps of aldosterone biosynthesis.

The activity of L- and T-type channels can be discriminated with the patch clamp technique thanks to the distinct electrophysiological properties of these channels. As T channels inactivate rapidly, L-type currents can be measured a few hundred milliseconds after depolarization, when T-type currents are completely abolished. In contrast, because L channels deactivate almost immediately upon cell repolarization, the tail currents elicited when cells are repolarized to −65 mV after a short period (20 ms) at positive voltages are exclusively due to T channel activity [9]. Moreover, some pharmacological drugs display partial selectivity for T- or L-type channel. For example, dihydropyridines, at sub-micromolar concentrations, reduce L-type currents without affecting T channel activity, while other drugs, like mibefradil, tetrandrine or zonisamide, are more efficient on T channels. Using this pharmacological approach, we

previously showed that the cytosolic calcium response observed upon glomerulosa cell stimulation with physiological concentrations of extracellular potassium is well correlated to L-type currents, but aldosterone production is clearly linked to T channel activity [10].

We have, therefore, proposed a model, in which calcium entering the cell through L channels is rapidly dispersed in the cytosol, while calcium entering through T channels is specifically conveyed to the mitochondria, where it stimulates aldosterone synthesis [1]. As T channels control aldosterone production, we wanted to know whether aldosterone itself can modulate T channel activity.

We observed that treatment of the human adrenal cortex H295R cells for 24 h with 1 μM aldosterone leads to a significant increase (by approximately 40%) of the T-type current density at the cell surface. Interestingly, the same treatment had no effect on the L-type currents. Moreover, aldosterone action on T-type currents was completely abolished by the MR antagonist spironolactone, which even slightly increased the L-type current density.

This effect of aldosterone on T-type currents was not due to a modification of T channel properties. Indeed, the activation and inactivation curves were similar in control cells and in cells treated with aldosterone, only the amplitude of the current was greater after treatment. This observation suggested an effect of aldosterone on T channel expression.

Seven different genes code for the main subunit of the various T and L channel isoforms, all that share a common general structure (Fig. 1). The core of these channels is made of a large protein of more than 2000 amino acids, α_1, composed of four repeats, each made of six transmembrane domains and linked by large intracellular loops. Three different genes code for T channels: α_1G, α_1H and α_1I, the latter being essentially expressed in neurons, while four genes code for L channels, α_1C, α_1D, α_1S (the skeletal muscle form) and α_1F (exclusively present in the retina). To determine which of these isoforms are expressed in adrenal H295R cells, we performed RT-PCR with specific primers designed in the second intracellular loop, a region of α_1 highly specific for each channel isoform (Fig. 1).

We found that, in normal culture conditions [5], only α_1H, α_1C and, α_1D are expressed in H295R cells. Interestingly, replacing ultroser, a commercially available serum, by fetal calf and horse serum in the culture medium, or removing the sera for 24 h, markedly increased the level of α_1G expression (Fig. 2).

By real-time RT-PCR, we observed, in response to aldosterone (1 μM, 24 h), a significant increase of α_1H mRNA levels, in the same proportion as the rise observed for the T-type current amplitude. Once again, spironolactone prevented aldosterone action. In contrast, α_1G was not affected by aldosterone.

Aldosterone effect on α_1H was maximal between 24 and 48 h, but no significant response was observed within the first 6 h, excluding a rapid, non genomic action of the steroid. In contrast, no significant effect of spironolactone alone was observed during the same period of time.

To determine the physiological relevance of this channel regulation, we have measured pregnenolone formation, during a one hour incubation, and this at various times during the treatment with aldosterone. While the basal steroid production did not change during this period, the response to potassium was clearly potentiated in parallel to the channel expression. In contrast, the response to ACTH, which stimulates steroidogenesis through the cAMP pathway, in a calcium-independent fashion, was unaffected by the treatment with aldosterone.

Finally, to further support a genomic action of aldosterone, we demonstrated by conventional RT-PCR that the MR is effectively expressed in H295R cells. However, because of the

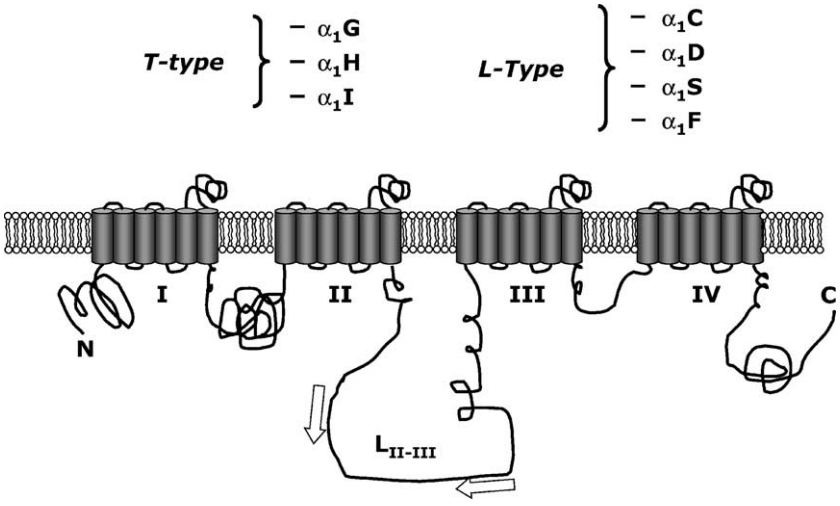

Fig. 1. Molecular structure of calcium channel α1 subunits. Voltage-operated calcium channel isoforms display a common structure of their main (α_1) subunit, with four repeated membrane domains linked by large intracellular loops. $L_{II–III}$: cytosolic loop linking domains II and III. Arrows indicate the approximate position of the specific primers used for PCR amplification. The three T-type and four L-type channel isoforms are also indicated.

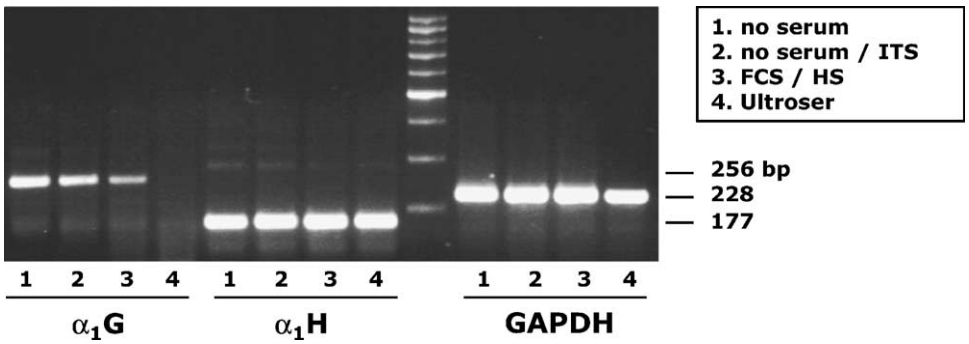

Fig. 2. Effect of culture conditions on α1G expression in adrenal H295R cells. Adrenal H295R cells were cultured as previously described [5], but were maintained for 24 h in the absence of serum, before being incubated for an additional period of 24 h, either: (1) in the absence of any serum, (2) in the absence of serum but in the presence of 1% ITS Plus, (3) in the presence of 2% FCS and 10% HS, or (4) in the presence of 2% ultroser. Total RNA was then extracted and reverse transcribed using random hexamers, before being amplified by PCR with primers specific for α_1G, α_1H and GAPDH. PCR products were then analyzed by gel electrophoresis. The predicted size of each amplicon is indicated on the right.

high concentration of aldosterone used in this study (1 μM), the presence of mRNA coding for MR does not exclude a possible action of the steroid through the glucocorticoid receptor, also present in these cells. Indeed, in preliminary experiments, we found that, not only aldosterone action is partially mimicked by dexamethasone, but it is also abolished by the glucocorticoid antagonist mifepristone (RU-486). This observation suggests that the glucocorticoid receptor could be involved in the regulation of T channel expression.

In summary, all these results together indicate that calcium flowing through T channels specifically activates aldosterone formation and secretion. However, the same hormone can activate, in a paracrine, or even intracrine manner, a receptor controlling the expression of additional T channels. The resulting increase of T channel activity, therefore, represents a positive feedback loop for the secretion of aldosterone in H295R cells (Fig. 3).

3.2. Aldosterone action on cardiomyocyte T channels

Aldosterone has been implicated in the pathogenesis of ventricular hypertrophy and heart failure, independently of its action on arterial blood pressure. This steroid has been shown to be produced by the heart itself and cardiac steroidogenic enzymes (like aldosterone synthase) are upregulated upon myocard infarct [11]. Moreover, the RALE study has clearly demonstrated a beneficial action of spironolactone on the survival of patients with severe congestive heart failure [12], and several groups have shown that aldosterone in vivo induces heart hypertrophy and fibrosis, and can lead to cardiac arrhythmias.

Nevertheless, the exact molecular mode of action of aldosterone on the cardiac function remains unclear. Recently, Bénitah and Vassort have shown that aldosterone in vitro increases cardiomyocyte calcium currents in the adult rat [13].

We observed a similar action of aldosterone on calcium currents recorded in cultured ventricular cardiac cells isolated from neonate rats. Indeed, after 24 h in culture with 1 μM aldosterone, the mean current amplitude was significantly increased (by 100%) at each tested potential. Aldosterone did not affect current kinetics or voltage-dependency.

After birth and during the adult life, L-type currents are the major currents present in rat cardiomyocytes. Aldosterone essentially affected L-type currents, without significant effect on T-type currents, which are responsible for <10% of the total calcium current.

Interestingly, aldosterone had apparently no effect on the currents recorded from atrial cells, an observation that has to be considered in relationship with the fact that cardiac ventricle is much more prone than atrium to remodeling-associated pathologies.

As expected, when quantified by real-time RT-PCR, the levels of α_1C mRNA (coding for L-type channels) appeared

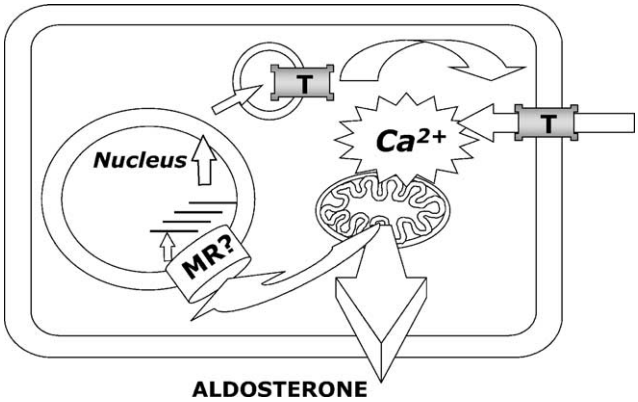

Fig. 3. Positive feed-back loop of aldosterone on its own biosynthesis in H295R cells. According to our model, calcium entering H295R cells through T channels is specifically conveyed to the mitochondria, where it activates early steps of aldosterone biosynthesis. The steroid is then immediately secreted by free diffusion across the biological membranes, but a part of it stimulates, in an intracrine manner, the mineralocorticoid receptors (MR) present in these cells. This stimulation results in increased T (α_1H) channel expression and, therefore, in increased steroidogenesis. Additional, calcium channel-independent mechanisms are nevertheless believed to prevent excessive aldosterone production [5].

markedly increased in ventricular cells after 48 h treatment with aldosterone. However, T channel isoforms also appeared affected by aldosterone. Indeed, while aldosterone slightly increased α_1H mRNA levels, it reduced those of α_1G, an isoform that is also expressed in these cells. This opposite effect of aldosterone on these two T channel isoforms could explain the low global effect of the hormone on T-type currents. Moreover, if the role of each of these two isoforms is distinct in cardiomyocytes (as it is probably the case) this complex modulation exerted by aldosterone could have specific consequences on the normal heart function.

3.3. Aldosterone action on prostate cell T channels

Neuroendocrine differentiation of prostate epithelial cells is usually associated with an increased aggressivity of tumors and a poor prognosis for cancer evolution. Cyclic AMP treatment of prostate LNCaP cells in vitro induces their neuroendocrine differentiation, with the appearance after 3 days of neurite-like structures. In collaboration with Prevarskaya and coworkers, we have recently shown that these morphological changes are accompanied with the expression of various specific markers, including T-type calcium channels [6].

Indeed, after treatment with cAMP analogs, the basal inward calcium current present in some LNCaP cells was significantly increased. The rapid inactivation of this current and its pharmacological properties were in agreement with a current supported by T-type calcium channels. On average, the current was increased by 100–150% after 3 days in the presence of 8-bromo- or dibutyryl-cAMP, and this effect was potentiated when cAMP was combined with the cAMP phosphodiesterase inhibitor, IBMX. Not only the amplitude of the current was increased in response to cAMP, but also the percentage of cells displaying this current, that rose from 20 to 60%.

By conventional RT-PCR, we showed that the α_1H and only this isoform among the various L- and T-type channels is expressed in control or cAMP-treated LNCaP cells. By real-time RT-PCR, we found a nice correlation between the levels of channel expression and their activity.

In order to determine a possible role for T channels in LNCaP cell differentiation, the same treatment was then performed in the presence of nickel, that blocks T channel activity, but not their expression. The length of neurites, measured under these conditions, appeared significantly reduced, suggesting a physiological role for calcium entering through T channels in cell differentiation.

It is well known that the sensitivity of prostate cells to ambient steroids is changing during cancer evolution. In vitro, we found that steroid depletion by itself leads, after 5–6 days, to LNCaP cell neuroendocrine differentiation and T-type current overexpression [6]. Because of this modulation, exerted by steroids present in culture media, and because it has been recently reported that cAMP can activate the MR in a ligand-independent manner [14], we hypothesized that the expression of T channels in LNCaP could be, at least partially, under the control of the MR. We found, by RT-PCR, that not only the MR is expressed in LNCaP cells, but also the type 2 11-β hydroxy-steroid deshydrogenase enzyme, that protects this receptor against activation by glucocorticoids. In contrast, the glucocorticoid receptor was not detected in this cell line. This observation, therefore, incited us to test the effect of aldosterone on T channel expression in LNCaP cells. However, in comparison to cAMP, aldosterone, after 24 h, appears to have only a weak effect on α_1H expression and does not potentiate the response to cAMP. Nevertheless, further experiments are still required, for example, to determine the time course of aldosterone action in these cells.

3.4. Perspective and conclusion

In addition to the rapid modulation of calcium channel activity exerted by hormones through kinases and G proteins, a slower but more sustained control on calcium influx is achieved by changing the level of a specific channel isoform expression. Aldosterone appears to exert its influence on an important class of cellular calcium gate, the T-type calcium channel. The physiological consequence of such a modulation will clearly depend on the functional role played by these channels in each cell type. While such functions have been proposed in several tissues, like controlling steroidogenesis in adrenal glomerulosa cells, regulating the pacemaker activity (and possibly inducing arrhythmias in pathological states) in cardiomyocytes, and participating to the neuroendocrine differentiation of the prostate epithelial cells, their exact role in these and other cells needs to be confirmed by directly interfering with their expression. For this purpose, approaches using small interfering RNA or transgenic animals should be proved very useful in the next future. A better comprehension of the specific function of each channel isoform in a given cell is indeed mandatory for predicting the consequence of complex modulations of several channels, as those exerted by aldosterone in adrenal or cardiac cells.

Another point that remains to clarify in the action of aldosterone on calcium channels is the relative involvement of the mineralocorticoid and of the glucocorticoid receptors. In this regard, the use of more selective MR antagonists, as well as the investigation of cells lacking the glucocorticoid receptor, like LNCaP cells, should help to precise the role of these receptors. The physiological relationship between glucocorticoids and mineralocorticoids varies from one organ to the other. Indeed, while glucocorticoids exert a protective effect in the heart, possibly in part by preventing aldosterone action through its receptor, the same hormones, if not locally inactivated by a protective enzyme, would mimic aldosterone effect on the kidney, inducing sodium retention, hypokalemia and hypertension. What determines the specificity of the steroid response in each tissue, being the receptor accessibility, the presence of receptor co-modulators or additional pathways, remains to be clearly demonstrated.

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