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Calcium oscillations triggered by cardiotonic steroids

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Na^+, K^+–ATPase (NKA) is well known for its function as an ion pump. Studies during the last decade have revealed an additional role for NKA as a signal transducer. In this brief review, we describe how cardiotonic steroids, which are highly specific NKA ligands, trigger slow Ca^{2+} oscillations by promoting the interaction between NKA and the inositol trisphosphate receptor, and how this Ca^{2+} signal activates the NF-κB subunit p65 and increases the expression of the antiapoptotic factor Bcl-xL. The potential tissue-protective effects of this signal are discussed.

Na^+,K^+–ATPase, a receptor for cardiotonic steroids

Na^+,K^+–ATPase (NKA) [1] belongs to the family of P-type ATPases and is a ubiquitous integral plasma membrane protein expressed in all eukaryotic cells. NKA uses the energy from ATP hydrolysis to export three Na^+ ions from the cell and to import two K^+ ions into the cell against an electrochemical gradient [2,3].

The pumping function of NKA is essential for the regulation of cell ionic content, pH and for maintaining resting membrane potential. NKA is a heterotrimeric protein complex consisting of a large catalytic α subunit, a heavily glycosylated β subunit and a tissue-specific regulatory subunit belonging to the FXYD proteins family. In mammals, there are four α subunits, three β subunits and seven FXYD [4].

The cardiotonic steroids (CTS) are highly specific NKA ligands that bind to all catalytic α-isoforms [5,6]. The CTS consist of a steroid core with a lactone ring and a sugar moiety. The CTS can be divided into two families, the cardenolides, to which ouabain and digoxin belong and the bufadienolides, to which marinobufagenin belongs. Ouabain, digoxin and marinobufagenin have been identified in human plasma. The cardenolides have a five-membered lactone ring and the bufadienolides a six-membered lactone ring. Ouabain, which is perhaps the best studied CTS, binds to the α subunit on the extracellular side in the cavity in the transmembrane domain at the interface created by six transmembrane segments αM1–6. Its lactone ring is buried within the transmembrane domain and

Abbreviations
CTS, cardiotonic steroid(s); Ins(1,4,5)P_3, inositol trisphosphate; Ins(1,4,5)P_3R, inositol trisphosphate receptor; NKA, Na^+,K^+–ATPase; Src, Src kinase.

the sugar moiety facing the extracellular side [7,8]. Recent studies have indicated differences in the capacity of the CTS to bind to the NKA catalytic subunit because of the number and nature of various sugar residues and changes in the position of hydroxyl groups of the steroid core [9].

The CTS are now generally considered as mammalian hormones. Studies using NMR and MS techniques have convincingly shown that ouabain, digoxin and marinobufagenin are present in human plasma and urine, bovine adrenal gland and hypothalamus, and rat adrenomedullary glands [10]. Their biological significance has been discussed for many years. CTS dose-dependently inhibit the activity of NKA. However, there is little support for the notion that CTS act as natriuretic hormones, because their circulating concentrations are most likely too low to induce an inhibitory effect on NKA ion transport. During the last decade, reports from several labs have revealed an additional role for NKA as a signal transducer [11–14]. Studies from our group have shed new light on the potential function of endogenous CTS as triggers of highly regular Ca^{2+} oscillations within a period range of 3–5 min.

Cardiotonic steroids trigger calcium oscillations

Several years ago our group reported that ouabain, in doses causing only partial NKA inhibition, acts as an inducer of regular, low-frequency intracellular Ca^{2+} oscillations that elicit activation of the transcription factor, NF-κB [15]. The study was performed on primary rat renal epithelial cells. This first unexpected finding was followed up by several studies on the role of the inositol 1,4,5-trisphosphate receptor [Ins(1,4,5)P_3R] in the generation of ouabain-triggered Ca^{2+} oscillations and the downstream effects of this signal pathway. Most of these studies were performed on COS-7 cells, a cell line derived from embryonic monkey kidney cells.

To examine whether the generation of the ouabain-induced Ca^{2+} oscillations required the generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], cells were transfected with a construct encoding a hyperaffinity Ins(1,4,5)P_3 absorbent, 'an Ins(1,4,5)P_3 sponge' that has more than 1000-fold higher affinity for Ins(1,4,5)P_3 than Ins(1,4,5)P_3R, traps Ins(1,4,5)P_3 and abrogates Ins(1,4,5)P_3-induced Ca^{2+} release [16]. Because ouabain was found to trigger low-frequency Ca^{2+} oscillations in cells expressing the Ins(1,4,5)P_3 sponge, we concluded that the ouabain effect was at least partially independent of the generation of Ins(1,4,5)P_3 and we went on to explore the possibility that the ouabain-triggered Ca^{2+} oscillations might be initiated by direct interaction between NKA and Ins(1,4,5)P_3R. NKA coimmunoprecipitated with Ins(1,4,5)P_3R, and with the use of FRET measurements, a close spatial proximity between NKA and Ins(1,4,5)P_3R could be demonstrated, which was significantly enhanced in the presence of ouabain. The interaction between NKA and Ins(1,4,5)P_3R was found to be mediated by the N-terminus of the NKA catalytic α subunit and the N-terminus of the Ins(1,4,5)P_3R. The amino acid residues LKK in the α N-terminus tail, which are conserved in most species and all α-isoforms, were found to be essential for the binding. Ouabain-triggered Ca^{2+} signals were suppressed in cells overexpressing a peptide corresponding to the α-subunit N-terminal tail, but not in cells overexpressing a peptide in which the lysine-rich (LKK) motif had been deleted. It was concluded from these studies that NKA and Ins(1,4,5)P_3R can form a signaling microdomain that triggers Ca^{2+} oscillations [17]. It was later shown that ankyrin, which binds to the N-terminus of NKA and Ins(1,4,5)P_3R, acts as a stabilizing scaffolding protein within this signaling microdomain [18] (Fig. 1).

Taking into account the potential physiological and pharmacological role of NKA-triggered Ca^{2+} oscillations, it is important to consider whether this effect is specific for ouabain or can be extended to other CTS. In ongoing studies, we have tested whether the cardenolide digoxin and the bufadienolide marinobufagenin may also trigger Ca^{2+} oscillations. Both digoxin and marinobufagenin have been identified in human plasma. Figure 2 shows the effects of 100 nM ouabain, 100 nM digoxin and 100 nM marinobufagenin applied to COS-7 cells loaded with the Ca^{2+}-sensitive dye Fura-2AM. Results from these preliminary studies suggest that all tested CTS will trigger Ca^{2+} oscillations of similar frequency. Ouabain 100 nM has previously been found to cause ~ 10% inhibition of NKA activity in COS-7 cells [17]. Similar dose–response studies have not yet been performed on cells exposed to digoxin or marinobufagenin.

CTS stimulate Src-dependent tyrosine phosphorylation

Binding of CTS to NKA will also stimulate tyrosine phosphorylation. These observations, originally made by Xie and Askari at Toledo University [19,20], have since been confirmed by many laboratories. The tyrosine phosphorylation is mediated by the Src family of kinases, and coimmunoprecipitation and FRET studies indicate that the NKA catalytic α subunit and Src form a functional complex [21,22].

Several lines of evidence suggest that CTS activation of the NKA/Src complex causes transactivation of the

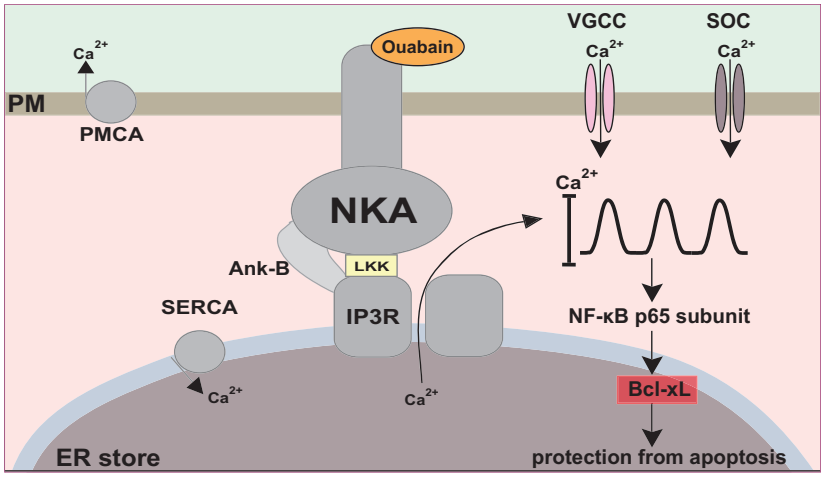

Fig. 1. NKA and Ca^{2+} signaling. Ouabain triggers the direct interaction between NKA and the Ins(1,4,5)P_3R. Amino acid residues LKK in the N-terminus of the catalytic α subunit of NKA are essential for binding with the Ins(1,4,5)P_3R N-terminus. This interaction is also supported by the scaffolding protein Ankyrin-B. The ouabain/NKA/Ins(1,4,5)P_3R complex will trigger slow Ca^{2+} oscillations that subsequently activate the NF-κB p65 and leads to protection from apoptosis. ER, endoplasmic reticulum; IP3R, inositol trisphosphate receptor; LKK, lysine-rich motif; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SOC, store-operated channels; VGCC, voltage-gated Ca^{2+} channels.

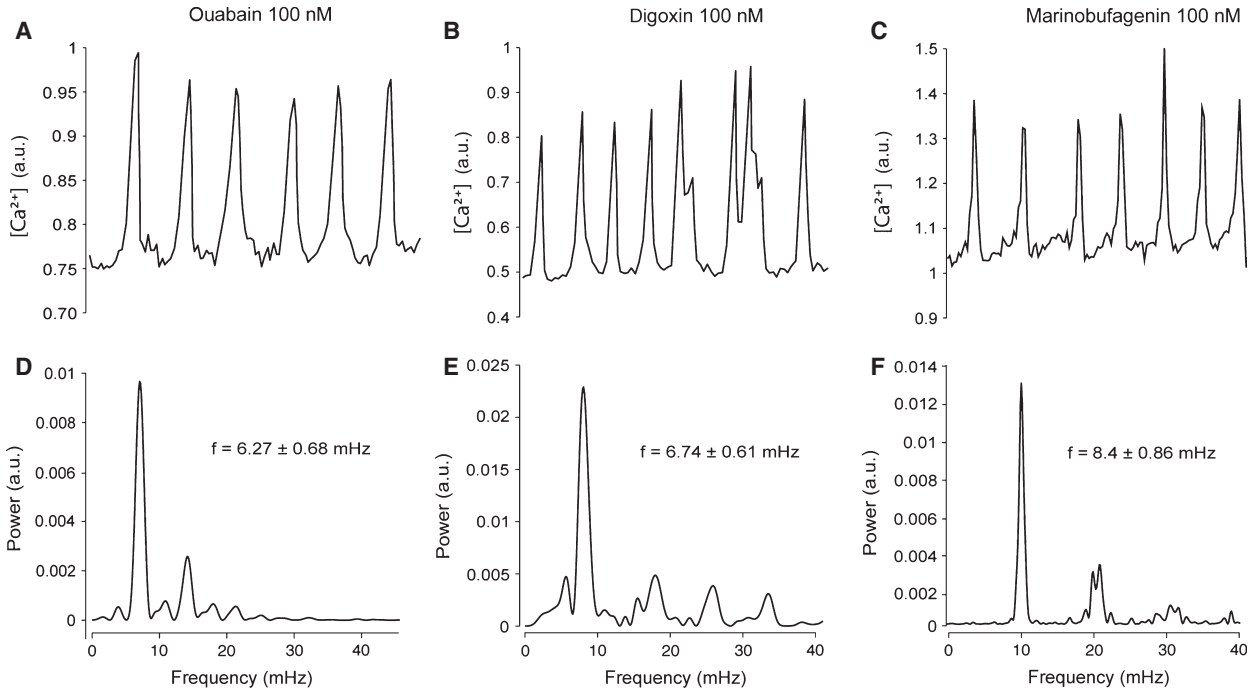

Fig. 2. Effect of ouabain-, digoxin- and marinobufagenin-induced Ca^{2+} oscillations in COS-7 cells. Cells were loaded with Fura-2AM and changes in $[Ca^{2+}]_i$ were recorded as a function of time after treatment with ouabain, digoxin and marinobufagenin. (A–C) Single-cell $[Ca^{2+}]_i$ tracings in response to the indicated ouabain, digoxin and marinobufagenin concentrations. Each plot corresponds to the single-cell recording above. (D–F) Power spectral analysis of the three CTS-evoked Ca^{2+} oscillations depicted in (A–C). For a methodological description see Zhang *et al.* [17]. Marinobufagenin was obtained from A. Y. Bagrov (National Institute of Health, Baltimore, MD, USA).

epidermal growth factor receptor and subsequent activation of signaling pathways downstream of the epidermal growth factor [21,23,24]. It has been suggested by Pierre and Xie that activation of Src may be the initiating event in downstream signaling invoked by interaction of the NKA with CTS [13]. In ongoing studies, we

haver tested whether Src phosphorylation is required for initiation of the CTS-evoked Ca^{2+} signaling pathway, and found that this is likely to be the case.

As shown in Fig. 3(A,D), pretreatment of COS-7 with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*]pyrimidine (PP2), an inhibitor of Src, significantly reduced the number of cells responding with Ca^{2+} oscillations. We have also tested the effect of inhibitors of signaling molecules that are known to be activated by the epidermal growth factor receptor, but to date have found no evidence for their involvement in the triggering of Ca^{2+} oscillations (Fig. 3B,C).

Downstream effects of calcium oscillations triggered by CTS

Ca^{2+} is the most versatile of all intracellular signals, because the cell can decode the amplitude and duration of the signal [25]. Studies in cell-free systems have indicated that Ca^{2+}-binding proteins, with multiple Ca^{2+}-binding sites, such as CaM kinase II, can decode the frequency of an oscillatory Ca^{2+} signal [26]. Reduction of the frequency of Ca^{2+} oscillations, accomplished by dose-dependent application of an Ins(1,4,5)P_3R inhibitor, has been shown to promote the activation of NF-κB transcriptional activity [27]. Many G_q-coupled receptors, including the metabotropic glutamate receptors, are known to trigger regular Ca^{2+} oscillations that generally have a higher frequency (0.5–1 min) than the Ca^{2+} oscillations triggered by CTS. Studies from our group have shown that the slow Ca^{2+} oscillations triggered by CTS activate the NF-κB survival factor p65 in the nucleus and increase its transcriptional activity [28,29]. p65 is known to control the transcription of the antiapoptotic factor Bcl-xL.

Antiapoptotic effects of ouabain/NKA/Ins(1,4,5)P_3R signaling have been demonstrated in developmental programming of kidneys exposed to malnutrition [30], and in kidneys exposed to Shiga toxin, a well-known cause of apoptosis [29]. Organ development requires a well-controlled balance between proliferation, differentiation and apoptosis. Explant embryonic kidneys were studied with regard to the level of apoptosis and nephron formation, and malnutrition was mimicked by serum starvation. This caused a robust increase in apoptotic rate and retardation of nephron formation. Exposure to 10 nM ouabain during the serum starvation rescued the cell from apoptosis and retarded nephron formation. The effects of ouabain were abolished in the presence of a p65 inhibitor and following depletion of Ca^{2+} stores in the endoplasmic reticulum. Ouabain (10 nM) had no measurable effect on the NKA pumping function. The intracellular sodium concentration was maintained at a constant 5 mM level. *In vivo* studies of nephron endowment in offspring of rats that received low-pro-

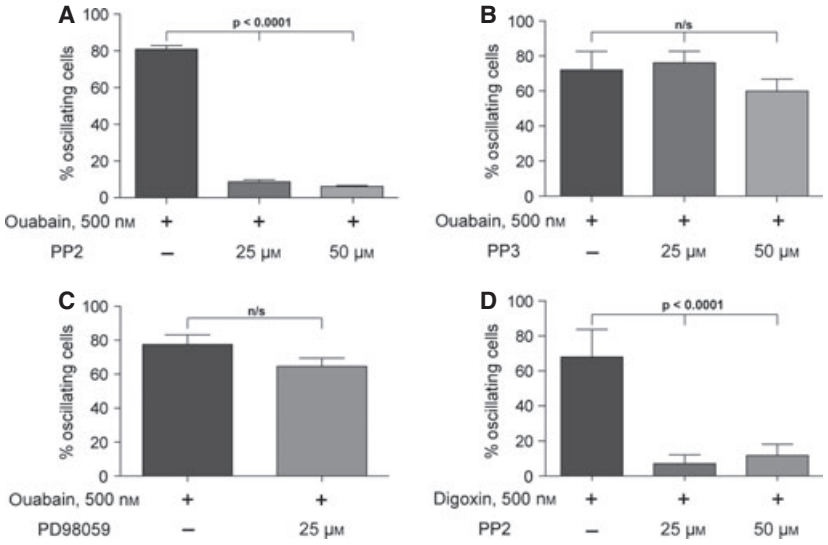

Fig. 3. Effects of one or more kinase inhibitors on ouabain- or digoxin-induced $[Ca^{2+}]_i$ oscillations. Cells were prepared as described in Fig. 2. Data are the mean ± SEM of the percentage of cells oscillating from at least three independent experiments, and the figure shows a representative recording of both the control and cells pretreated with different concentrations (25 and/or 50 μM) of inhibitor. (A) Ouabain-induced Ca^{2+} oscillations are significantly suppressed by the Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP2), but not by its inactive equivalent 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (B) or the ERK 1/2 inhibitor 2′-amino-3′-methoxyflavone (PD98059) (C). (D) Digoxin-induced Ca^{2+} oscillations are significantly suppressed by PP2.

tein diet during pregnancy and were treated with either vehicle or ouabain confirmed the results from the *in vitro* studies. The metanephric mesenchymal cells that are about to differentiate into primitive nephrons exhibited Ca^{2+} activity that was enhanced by ouabain in both acute and chronic experiments.

Shiga toxin has a well-documented apoptotic effect. Shiga toxin is produced by the strain of *Escherichia coli* that causes hemolytic uremic syndrome. Shiga toxin binds to kidney epithelial cells and neurons and is a major contributor to loss of renal function and cognitive difficulties following the acute disease. Shiga toxin acts on the proapoptotic factor Bax to promote the intrinsic mitochondrial pathway [31]. Ouabain, in concentrations that should have little or no effect on the NKA pumping function in studies on rat primary renal epithelial cells and *in vivo* studies on mice, was shown to protect against Shiga-toxin-triggered apoptosis by upregulating Bcl-xL and downregulating Bax.

The downstream effects of ouabain-triggered Ca^{2+} oscillations cannot be attributed to inhibition of the NKA pumping function [17,28]. In acute experiments, the threshold concentration of ouabain required for triggering Ca^{2+} oscillations gives < 10% inhibition of Rb^+ uptake in COS-7 cells. If cells are exposed to 10–50 nM ouabain for several hours, Ca^{2+} oscillations are observed in ~ 5–30% of COS-7 cells [17]. Ouabain does not induce Ca^{2+} oscillations in cells in which the endoplasmic stores of Ca^{2+} are depleted. Treatment of COS-7 cells for 24 h with 1 nM ouabain activates the NF-κB p65 subunit and gives complete protection from apoptosis induced by serum starvation.

The plant-derived cardenolide digitalis has been used for more than a century to treat cardiac disease, and it seems likely, although is yet not proved, that at least some of the beneficial effects are related to the signaling function and tissue preserving effects of the CTS/NKA signaling. The cardiovascular effects of the CTS in humans are, however, somewhat controversial. Recent studies have drawn attention to the relationship between elevated serum values of the bufadienolide and increased risks of cardiovascular complications in end-stage kidney disease [32,33]. We speculate that the adverse effects of CTS may to some extent be related to Ca^{2+} homeostasis. There are reports indicating that digitalis toxicity is most commonly observed in patients with hypercalcemia [34]. Hypercalcemia related to hyperparathyroidism is a common complication in patients with end-stage kidney disease and an experimental study that showed the adverse cardiovascular effects of high circulating levels of marinobufagenin was performed on rats with severe kidney insufficiency and documented hyperparathyroidism [35].

Summary and perspectives

We have presented evidence for a signaling function of NKA that is activated by CTS and that involves the generation of slow Ca^{2+} oscillations, activation of the NF-κB survival factor p65 and generation of the antiapoptotic factor Bcl-xL, which counteracts the intrinsic mitochondrial apoptotic pathway. Evidence is also presented for a tissue-protective effect of NKA-generated Ca^{2+} oscillations during adverse developmental programming and following exposure to bacterial toxins. Important topics for future studies will be to clarify by which mechanisms Ca^{2+} oscillations activate p65 and to investigate how NKA Ca^{2+} signaling is related to mitochondrial function.

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