Chapter

Influence of Cardiac Glycosides and Prostaglandins on the Physiology of Epithelial Cells

Arturo Ponce, Liora Shoshani, Alejandro Ogazon del Toro and Marcelino Cereijido

Abstract

Epithelial cells play a major role in animal and human homeostasis because they selectively regulate the exchange of solutes between two given media, such as blood or urine. Cardiac glycosides (CG) are a group of highly toxic compounds whose best therapeutic known effect is on heart, although recent evidence has shown that it exerts a wide range of physiological effects on cells and tissues other than the heart. Prostaglandins, on the other hand, are a group of lipids that produce diverse physiological and pathological effects among which inflammation stands out. In this chapter, we describe that cardiac glycosides modulate key features of epithelial cell physiology, including cell-cell contact junctional complexes, cilliogenesis, and gap junction-mediated intercellular communication (GJIC) in epithelial cells. Prostaglandin PGE2 also modulates GJIC through a different signaling pathway. In addition, we describe that CG induce paracrine release of prostaglandin PGE2, which in turn modulates GJIC by itself.

Keywords: epithelia, gap junctions, prostaglandins, cardiac glycosides, Na (+)/K (+) ATPase

1. Introduction

Cardiac glycosides are a group of compounds with toxic and therapeutic, properties, best known for their effect on cardiac muscle, among which ouabain, digoxin, and marinobufagenin stand out. These compounds are produced by plants and a few animal species as defense mechanism. The fact that some of them are produced endogenously by some mammal species, including human beings, has led to consider them a new class of hormones, although the functions they play are still largely unknown.

We have studied the effect that several cardiac glycosides (ouabain, digoxin, and marinobufagenin) produce in the physiology of epithelial cells. As we describe in more detail later, we have found that they induce changes on key processes and structures, including cell-cell contacts, apical/basolateral polarity, and the expression of ion channels. In this chapter, we first, briefly, describe the properties of epithelial cells, as well as cardiac glycosides and prostaglandins. Then, we describe the results

showing that CG modulate some epithelial properties. Particularly, we address the finding that some cardiac glycosides (ouabain, digoxin, and marinobufagenin) modulates gap junctional-mediated intercellular communication (GJIC) since it led us to discover that prostaglandin E2 (PGE2) also modulates GJIC. Besides that ouabain also induces paracrine release of prostaglandin (PGE2), which acts synergistically to enhance the effect caused by ouabain on GJIC.

2. Epithelial cells, cardiac glycosides and prostaglandins. Basic concepts

2.1 Epithelial cell properties

The leading role of epithelial tissues is to separate two biological compartments with different chemical and physical properties, selectively allowing the exchange of substances between them. For this reason, epithelial cells have two fundamental features: (1) They establish contacts with their neighboring cells to form a physical barrier, and (2) their membrane is polarized; that is, the composition and properties of the membrane domain that faces one compartment are different from the membrane domain that faces the other compartment.

As schematized in **Figure 1**, vertebrate epithelial cells build three types of molecular complexes related to cell-cell contact (tight junctions, anchoring junctions, and gap junctions), which differ from each other in function and molecular composition [1]. Tight junctions form barriers, constituted as continuous, anastomosing strands between two cells [2]. Their function is to regulate the flow of solutes through the epithelium outside the cells (paracellular route) as well as promote and maintain apical/basolateral

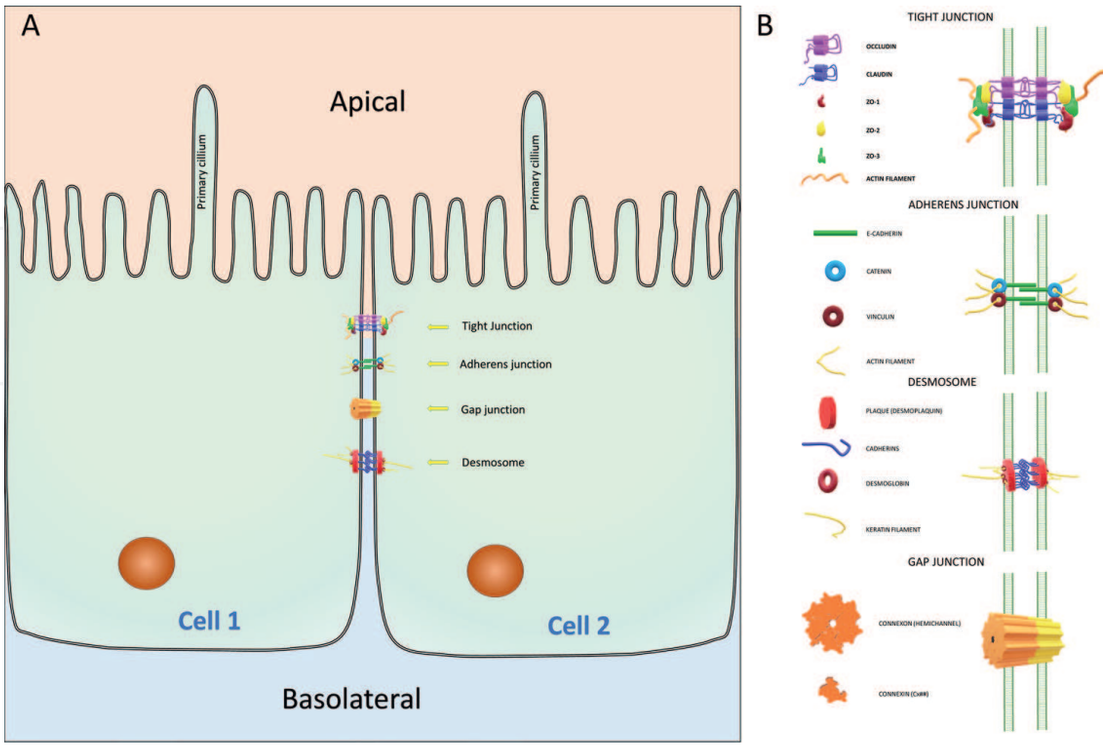

Figure 1.
Molecular structures of contact between epithelial cells. Part A schematizes two epithelial cells and their apical and basolateral membrane domains, as well as the various types of contact between them. Part B shows in greater detail each structure as well as their molecular components.

membrane polarity. Tight junctions are composed of claudins, tetra-spanning membrane proteins, whose extracellular domains are linked correspondingly, like a zipper, between the two connected cells. In turn, its intracellular domain is linked to a variety of proteins, including ZO-1, ZO-2, and ZO-3 that act as scaffolds, connecting TJs to the cytoskeleton. In addition to claudins, other integral membrane proteins such as tetra spanning membrane protein occludins and immunoglobulin superfamily proteins including junctional adhesion molecules (JAMs) localize to TJs.

Anchoring junctions provide mechanical stability and link cells to each other or to the extracellular matrix. There are two subtypes of anchoring junctions, adherens junctions and desmosomes. Adherens junctions form a continuous belt around contacting cells, while desmosomes are spot-like structures which, like rivets, reinforce the stability of the cell-cell connection [3]. The major transmembrane protein of the adherens junction is cadherins (most commonly E-cadherin). Nectins initiate intercellular contacts through trans-pairing with nectins on opposing cells and then recruit E-cadherin to these sites, facilitating the E-cadherin-E-cadherin contact [4]. Cadherins are structurally linked with several types of cytoplasmic proteins, including catenins and vinculins, which in turn connect with actin filaments that locate like a belt, along the inner perimeter of the cells. Desmosomes are also made up of multiple proteins: Desmosomal adhesion molecules (desmoglein and desmocholine) are transmembrane proteins, whose external domain provides the link between cells, while the cytoplasmic side is connected, through intermediate proteins (desmoplakines) with intermediate filaments made of keratins [5].

Gap junctions are molecular structures coexpressed by neighboring cells of animal tissues. Through them, the cells actively communicate by exchanging electrical signals and small molecules, such as ions, metabolites, and peptides or nucleotides of low molecular weight. This property allows animal tissues to perform rapid and coordinated functions, among which some of the most notable examples are the electrical synapse between neurons and the synchronized secretion of export proteins or hormones in endocrine glands, such as the pancreas or salivary glands [6, 7].

Gap junctions consist of the conjunction of two hemichannels or connexons, each of them contributed by one of the communicating cells. In turn, each hemichannel is composed of protein subunits called connexins, arranged in the form of a hexamer. Cells typically express tens or hundreds of these communication channels, as clusters of varying sizes. There are a variety of connexins (Cx), which differ in the sequence of amino acids that constitute them. In the mouse genome, 19 connexin genes have been found and 20 in the human genome, which classically are named according to the molecular weight of the protein (Cx32, Cx40, Cx43, etc.). A given connexon can be made up of connexins of the same type (homomeric) or different connexins (heteromeric). Also, a given GJ can be made up of connexons of the same type (homotypic) or different (heterotypic) combinations of connexins [8].

Despite being overly complex structures, gap junctions are highly dynamic, that is, the connexins that integrate them are changed very quickly; thus, the number of gap junction units present between two given cells and determines the ease of communication between them, and this property is in turn dependent on several mechanisms and factors that modulate it.

2.2 Na (+)/K (+) ATPase: classic and new roles

Along with the cell-cell contact components described above, it is worth considering Na (+)/K(+) ATPase as an important player in epithelial physiology. Since its discovery

by Jens Christian Skou in 1957 [9], Na (+)/K(+) ATPase has grown in importance because it is a ubiquitous molecular component of virtually all types of cells whose most important and most widely studied function is as a pump, responsible for producing and maintaining the gradient of Na and K ions at the expense of energy expenditure. The pump consists of two main subunits: a α subunit and a β subunit. Subunit α is the catalytic subunit and contains the binding sites for sodium (Na+) and potassium (K+) ions, as well as the binding site for ATP. The β subunit is needed to stabilize the conformation of the α subunit and to assist in the insertion of the pump into the cell membrane [10]. In addition to its function as a pump, it has recently been described that Na (+)/K (+) ATPase can function as a signal transducer, induced by cardiac glycosides when these are at nonsaturating concentrations (in the pico or nanomolar range) [11]. In addition to this, the β subunit has been described to be a homophilic adhesion molecule [12, 13] that regulates cell polarity, cell motility, epithelial to mesenchymal transition, and oncogenic transformation. Several studies have shown that β-β interaction between epithelial cells is essential for the integrity of intercellular junctions [14].

2.3 Cardiac glycosides

As the name implies, cardiac glycosides (CGs) are a family of compounds that share similarities, both in their chemical structure and in some of the effects they produce, among which the effect on the heart of various species of mammals,

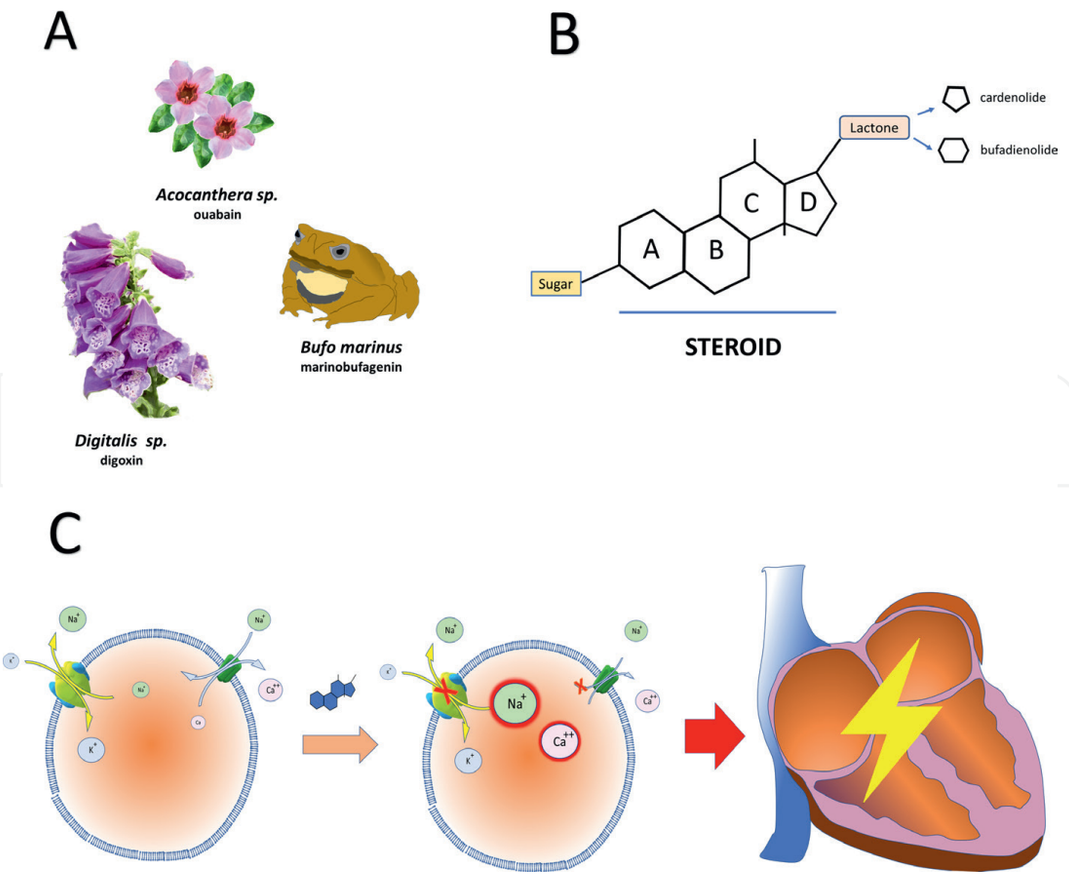

Figure 2.
Cardiac glycosides. (A) Most relevant species from which cardiac glycosides are obtained. (B) General chemical structure of cardiac glycosides. (C) Inotropic positive effect explains the increased contraction force in the heart.

including humans, stands out. A substantial number of different types of cardiac glycosides have been described, most of them synthesized by plant species, as a form of defense against predatory species, although a few species of animals, including toads, are also capable of producing cardiac glycosides in toxic quantities on their own (**Figure 2A**). Other animal species, such as monarch butterflies, have evolved to incorporate CGs into their bodies after consuming plant species that produce them as a defense strategy against predators [15].

Some of these compounds have been known empirically since ages by their toxic properties. Most remarkably, African tribes used extracts from leaves and roots of plants (*Strophantus gratus* and *Acokanthera schimperi*) to poison arrowheads for hunting and warfare purposes. Its therapeutic properties were first described by the Scottish physician and botanist William Withering in 1785, who observed that infusions of the foxglove plant (*Digitalis purpurea*) can cure dropsy, the edematous bodily swelling that typically accompanied heart failure [16, 17]. In 1930, digoxin was isolated and identified as the active compound of the foxglove plant [18].

The chemical structure of cardiac glycosides consists of a steroid ring, a lactone ring with five or six carbons, and a sugar moiety (**Figure 2B**). There are two classes of cardiac glycosides, cardenolides and bufadienolides, chemically distinguishable by the lactone ring. Cardenolides contain a five-membered ring with a double bond, while bufadienolides contain a six-membered ring with two double bonds [19].

Cardiac glycosides produce their therapeutic and toxic effects because they partially or totally inhibit the pumping function of Na (+)/K (+) ATPase. At therapeutic doses, this induces a positive inotropic effect; that is, an increase in the intracellular concentration of Na+, which in turn causes an increase in the intracellular concentration of Ca2+ by overloading of the Na+/Ca2+ exchanger which in turn causes the contractions of the cardiac muscle to be more sustained (**Figure 2C**) [20].

However, its therapeutic index is very narrow. For this reason, several of these compounds have been discontinued for medical purposes or are used only under strict medical supervision. Unexpectedly, it has been found that some cardiac glycosides (ouabain, marinobufagenin, bufalin, 19-norbufalin, and proscillaridin A) are synthesized endogenously by some species of animals, including humans, which has led to consider them hormones [21], although their physiological functions are yet mostly unknown.

2.4 Prostaglandins

Prostaglandins are a family of chemical compounds of great physiological, pathological, and medical interest due to the wide variety of effects produced by animal organisms, including humans. Prostaglandins, like hormones, are secreted to the extracellular environment and, after binding to receptors in the membrane of target cells, activate signaling cascades, which in turn produce changes in those cells. However, unlike hormones, prostaglandins are released to act locally, either in the same cell that produced them (autocrine) or in neighboring cells (paracrine). Unlike hormones, prostaglandins are not synthesized in a specific gland or tissue but are produced in virtually any tissue that constitutes an organism, and their production and secretion depend in turn on various stimuli to the cells, among which mechanical stress or the presence of other chemical substances stand out [22].

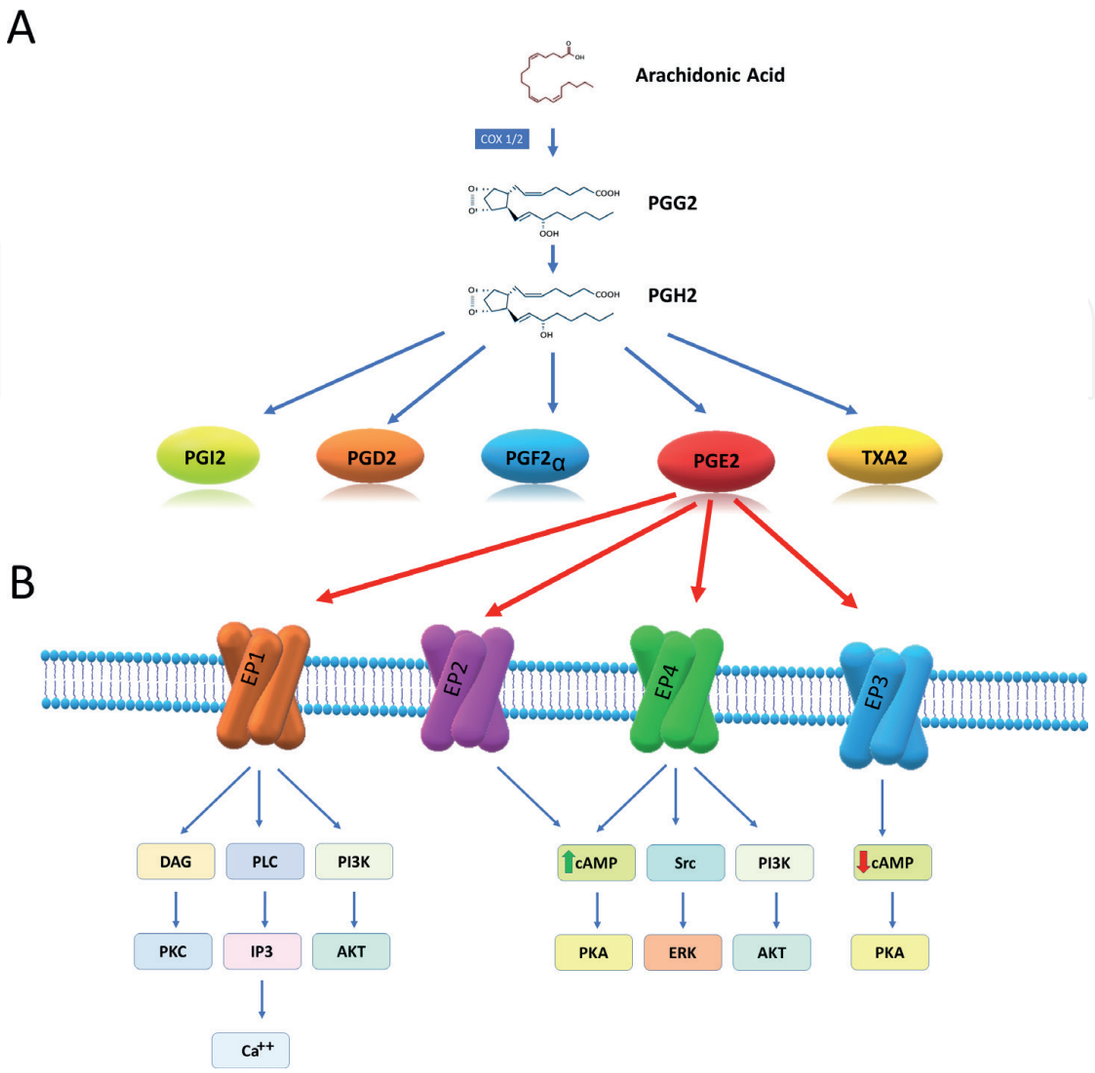

Figure 3.
Prostaglandins. (A) As shown all different types of prostaglandins are synthesized from arachidonic acid by distinct enzymatic steps. (B) Outlines the different prostaglandin E2 receptors as well as the different signaling pathways associated with each.

There are a variety of types of prostaglandins, and their effects depend on variations in their chemical structure, as well as the receptors they bind to on target cells. All of them are, however, similar in their chemical structure, lipidic in nature, with a molecular template consisting of a skeleton of 20 carbons, that includes a pentameric ring (cyclopentane) and two side chains, a carboxyl and an alkyl type. All distinct prostaglandins are produced from arachidonic acid, through several stages of enzymatic transformation (**Figure 3A**). The first stage involves cyclooxygenases 1 and 2 (Cox1 and Cox2) to produce PGG2 and PGH2, which are subsequently transformed by other enzymes to produce the different variants, classified into several subfamilies A, B, C, D, E, F, G., H, and I [23, 24], a classification that is based on the structure of the cyclopentane ring.

Prostaglandin receptors are membrane proteins coupled to G proteins. The various signaling pathways activated depend on the type of G protein subunit to which they are coupled, either Gs, Gi, or Gq [25]. The different types of prostaglandin receptors are named according to the type of prostaglandin subfamily to which they are most related, so, for example, the type E prostaglandin receptors are called EPs, while those of the D subfamily are called DP, and so on [26].

PGE2, the most abundant and widely produced prostaglandin, has four receptors (EP1, EP2, EP3, and EP4), which activate different signaling pathways. Thus, the Ep1 receptor activates the diacylglycerol, PLC, and PI3K pathways, while the EP2 and EP4 receptors increase the synthesis of cAMP, which can activate its effectors, mainly PKA, while the EP3 receptor has the opposite effect, inactivating the synthesis of cAMP. The EP4 receptor can also activate the PI3K/AKT and c-Src pathway, which can activate ERK in addition to transactivating other receptors such as the EGF receptor (**Figure 3B**) [27].

Cells do not express all receptors; instead, depending on the type of cell, they express one or two receptors as predominant forms. Therefore, PGE2 can have different effects on different cell types depending on the specific type of receptor that expresses [28]. Further, receptors are not entirely specific since they can bind other prostaglandins, although they do so with less affinity with respect to prostaglandin which is their main ligand. In addition, each receptor subtype may have a different affinity for its main ligand [29].

EP receptors have different expression patterns and are sometimes expressed constitutively; the most abundant are Ep3 and EP4 [30] as they are present in a large variety of tissues. In contrast, EP1 occurs only in a few tissues such as kidney, stomach [31], and nervous system [32]. EP2 is the least present in tissues but can be induced under different conditions [33].

3. Influence of cardiac glycosides and prostaglandins on epithelial cell-cell contacts

3.1 Effect of cardiac glycosides on epithelial physiology

Through assays on epithelial culture cells, we have demonstrated that ouabain and other cardiac glycosides, in a nanomolar range, influence various structures and components that have a fundamental role as intercellular contacts. In addition to this, they also influence several characteristics that have to do with the development of membrane polarity and transport. Most of these studies have been carried out using as a biological model MDCK, a cell line derived from dog kidney that in culture exhibits epithelial properties. After seeding, MDCK cells attach to the substrate, establish contacts between them, and eventually polarize its membrane into a baso-lateral domain, which faces the substrate, and an apical one that faces the culturing media [34].

3.1.1 Effect of ouabain on cell-cell contacts

Ouabain induces changes in the expression of proteins (claudins 1, 2, and 4) that constitute the intercellular molecular complex known as tight junctions (**Figure 4A**). These changes to the expression of claudins lead to augmenting the strength of sealing between cells, as evidenced by an increase in the transepithelial electrical resistance (TER) [35].

Ouabain also influences adherens junctions (**Figure 4B**), by enhancing the expression of some of its constituents (E-cadherin, β-catenin, and γ-catenin), a response involving Na (+)/K (+) ATPase as receptor and cSrc and Erk1/2 in the signaling cascade [36].

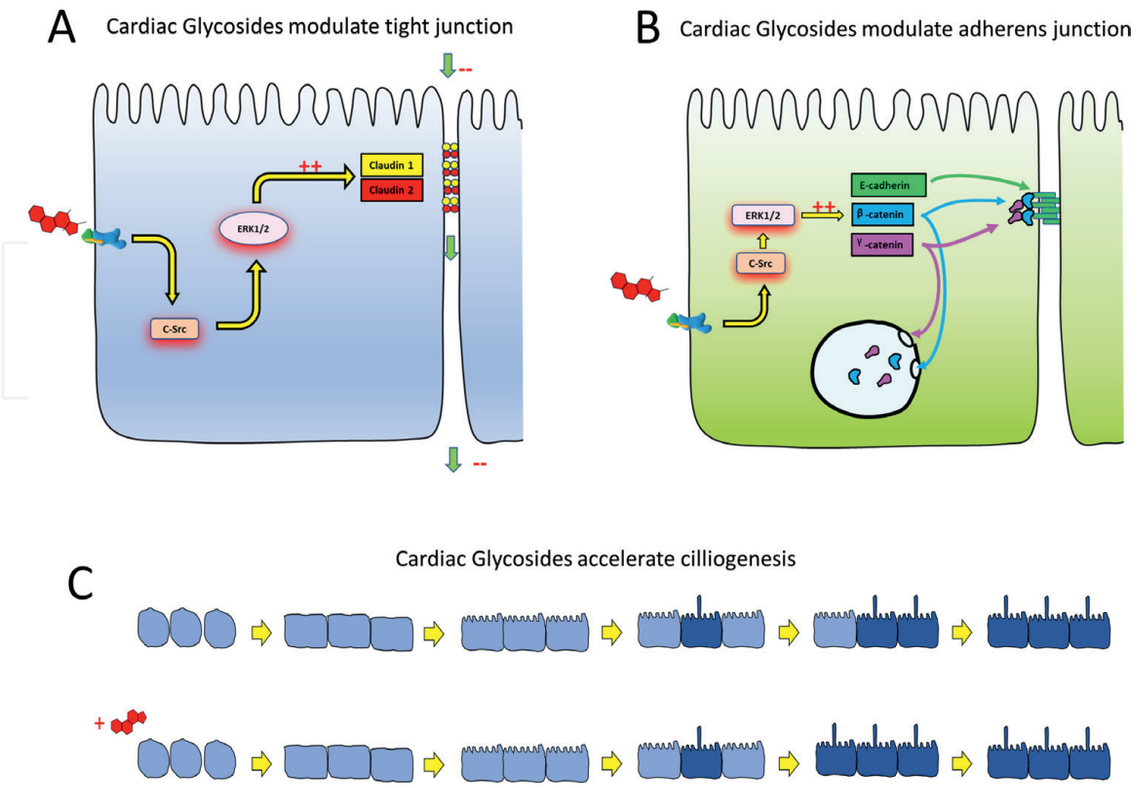

Figure 4.
Effect of cardiac glycosides on epithelial cells. (A) The binding of either ouabain, digoxin, or marinobufagenin to Na (+)/K (+) ATPase activates a signaling pathway, which includes cSrc and Erk1/2, and which promotes an increase in resistance to the flow of ions and small molecules through the paracellular pathway. This is due to an increased expression of claudins 1 and 4. (B) The binding of ouabain to Na (+)/K (+) ATPase, activates a signaling cascade that includes also cSrc and Erk1/2 and promotes enhanced membrane expression of E-cadherin and β and γ catenins. β and γ catenins are also sent to the nucleous. C) Ouabain increases the speed up the maturation of epithelial polarization, as evidenced by the fact that cell develop faster their primary cilium.

3.1.2 Ouabain speeds up the building of membrane polarity and ciliogenesis

Another interesting effect that ouabain produces in epithelial cells is to accelerate cilliogenesis. As is known, most epithelial cells possess a primary cilium, a hair-like process that protrudes from the center of the apical membrane and is thought to serve as flow sensor [37–39]. By immunohistochemical assays, we compared the percentage of MDCK cells that had developed the cilium, at different times after seeding at confluence, under either the presence or absence of ouabain, so we found that ouabain 10 nM induces the cells to develop their primary cilium faster than those without treatment (**Figure 4C**) [40]. These results suggest therefore that ouabain influences how quickly cells are polarized.

3.1.3 Effect of ouabain on the expression of voltage-gated potassium channels

We have also found, with electrophysiological procedures, that in MDCK cells arranged as mature monolayers, and ouabain enhances the magnitude of voltage-gated potassium currents (IK) and accelerates the recovery of IK in cells previously trypsinized and re-seeded at confluence. These ouabain-induced changes on IK require the synthesis of new nucleotides and proteins as well as Golgi processing and exocytosis [41].

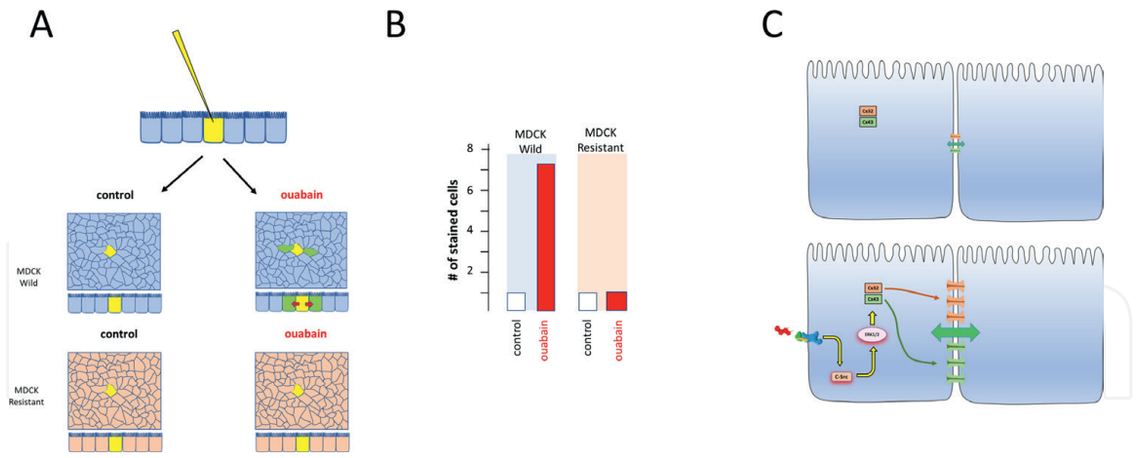

Figure 5.
Ouabain enhances gap junctional intercellular communication. (A) Dye transfer assays are performed by injecting a mixture of a permeant (neurobiotin-FITC) and a non-permeant dye (dextran-TRITC) to a single cell, chosen at random from a monolayer. (B) The average number of cells stained in green after injecting a single cell estimates GJIC, which ouabain increases notoriously in wild but not in resistant MDCK strains. (C) The binding of ouabain to Na (+)/K (+) ATPase activates a signaling pathway that includes the participation of cSrc and ERK1/2 and promotes the localization of connexins 32 and 43 to the membrane, resulting in an increase in intercellular communication mediated by gap junctions (GJIC).

3.2 Cardiac glycosides enhance GJIC

To find out whether ouabain and other cardiac glycosides influence the intensity of intercellular gap junction communication (GJIC), we performed dye transfer assays on MDCK cells arranged as mature epithelial monolayers. As outlined in **Figure 5A** and **B**, this technique consists of randomly choosing a cell from among those that make up the mature monolayer to inject it with a solution containing a mixture of two compounds: (1) dextran conjugated with TRITC, a compound that fluoresces in red and does not pass through gap junctions, and (2) neurobiotin conjugated with FITC, a compound that diffuses through the gap junctions and fluoresces in green. In this way, if there is communication between the injected cell and its neighbors, the neurobiotin-FITC diffuses and as a result green-stained cells are shown, next to the injected cell, which fluoresces yellow due to the mixture of red and green. If, on the other hand, there is no GJIC, only the injected cell fluoresces in yellow. Thus, the average number of green-stained cells, resulting from a set of trials, is an estimate of the degree of gap junctional communication between the cells in the monolayer. In this way, we have shown that in MDCK cells in mature monolayers, and ouabain (10 nM) induces a significant increase to GJIC significant from 20 minutes after having added ouabain to the extracellular. As also is schematized in **Figure 5A** and **B**, this response is not obtained in MDCK-R cells, a strain in which Na (+)/K (+) ATPase was modified by mutagenesis to render it insensitive to ouabain [42], demonstrating that Na (+)/K (+) ATPase is the receptor that, after the binding of ouabain triggers a signaling cascade involving cSrc and Erk1/2. Moreover, using silencing assays, we also demonstrated that this ouabain-induced enhancement of GJC involves connexins 32 and 43 [43].

This increase in GJIC does not require synthesis of new protein components because the inhibitors cycloheximide and actinomycin D do not affect this phenomenon. It rather involves the re-localization of Cnx43 subunits, already synthesized, to the plasma membrane [44]. Subsequently, using the same method of dye transfer in

MDCK, we have shown that digoxin and marinobufagenin, and other cardiac glycosides that have been described as endogenously expressed in mammals also induce a significant increase in GJIC [45].

3.3 Effect of prostaglandins on GJIC

Using the same experimental strategy (dye transfer) and the same biological model (MDCK cells), we evaluated whether PGE2 induces any effect on GJIC. We found that PGE2 induces a statistically significant increase in GJIC from 100 nM and from 15 min after its addition to the medium. This effect does not require the synthesis of new mRNA or proteins subunits but rather trafficking of subunits already synthesized. We also found, as depicted in **Figure 6**, that such effect is mediated by the E2 receptor, which, in turn, triggers a signaling pathway that includes activation of adenylyl cyclase and protein kinase A (PKA) [46].

3.4 Synergistic effect of cardiac glycosides and prostaglandins on GJIC

The finding that ouabain modulates GJIC in wild cells (MDCK-W) but not in resistant (MDCK-R), motivated us to do tests in mixtures of wild and resistant cells, to know if there could be cooperativity between both types of cells, since we had previously shown that at concentrations of ouabain in the micromolar range, there is metabolic cooperation among wild and resistant cells [47–49]. The results showed positively that under this condition, ouabain 10 nM induces MDCK-R cells to enhance GJICs. This led us to test further the possibility that prostaglandins could be the signal that enables resistant cells to increase their GJIC. For this purpose, we performed dye transfer assays in MDCK-W, MDCK-R cells, and mixtures of both cell types in various experimental conditions. So, we found that (1) the culture medium in which wild cells had been kept induces by itself an increase in GJIC in resistant cell monolayers. (2) This effect is abolished if wild cells are treated with COX inhibitors or if resistant

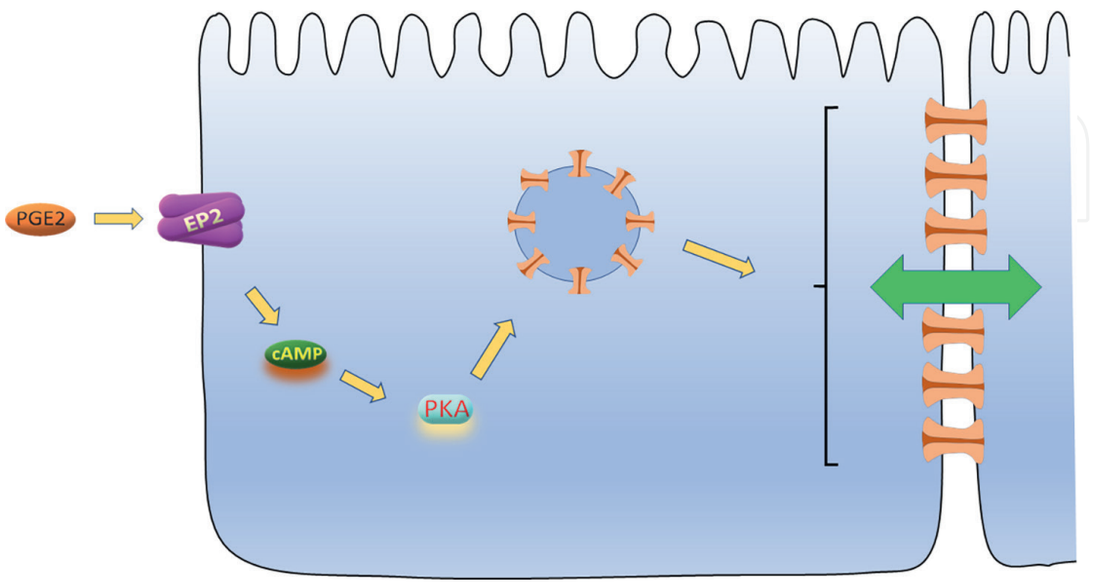

Figure 6.
Prostaglandin E2 enhances gap junctional intercellular communication. This drawing outlines the components associated with the enhancement of GJIC obtained by incubating MDCK cells in mature monolayer with PGE2, that includes the EP2 receptor, as well as adenylyl cyclase and protein kinase a (PKA).

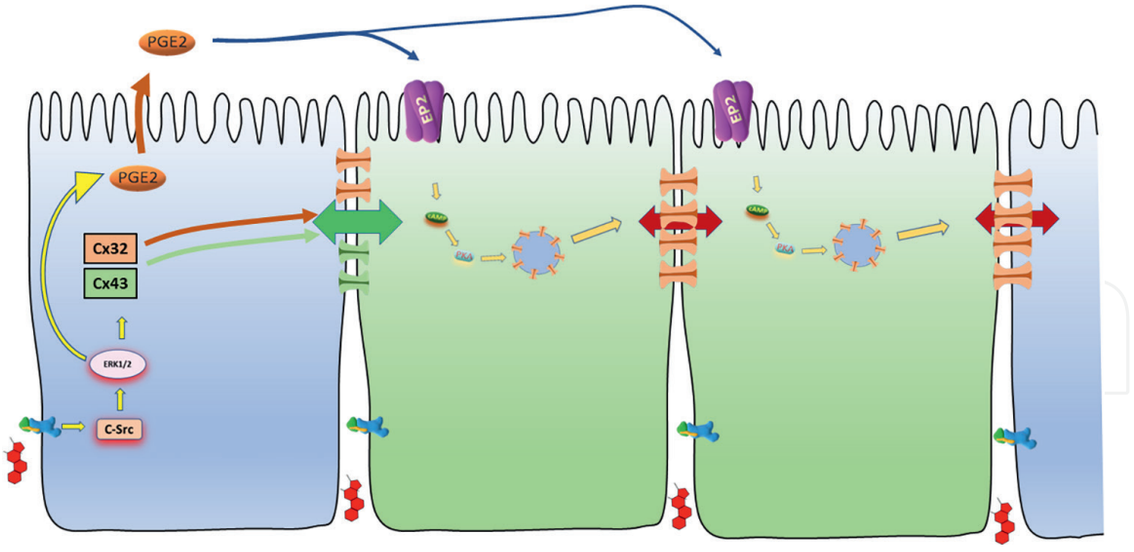

Figure 7.
Scheme representing the synergistic action of ouabain and PGE2 in GJIC in epithelial monolayers formed with the mixture of wild (in blue) and resistant (in green) cells. Both substances promote the activation of different signaling pathways as indicated therein. In wild cells, ouabain, in addition to promoting increased GJIC, also promotes the synthesis and release of PGE2 which then acts on neighboring cells as described in the text.

cells are treated with EP2 receptor blockers. These results then indicate, as outlined in **Figure 7,** that in MDCK-W cells ouabain, in addition to inducing an increase in GJIC, in the way described above, also induces the synthesis and release of prostaglandin E2 to the extracellular medium, which binds to the EP2 receptors of MDCK-R cells present in the mixture in culture, inducing an increase in GJIC in the way that has been described above, further creating a synergistic effect on MDCK-W cells [50].

4. Conclusions

Cardiac glycosides and prostaglandins are themselves chemical compounds of great relevance, although their functions are still largely unknown, both physiologically and pathologically. The results described here, which indicate a synergistic action in intercellular communication mediated by gap junctions, throw an additional interest and could lead to explain several of the effects that together or separately play both types of substances, even more could lead to a new strategy for the use of these compounds as therapy to alleviate diseases in which communication mediated by gap junctions is compromised. We do not know so far if this synergistic action is limited only to GJIC ls in epithelia or if it also extends to other types of intercellular contact. Nor do we know if it is limited to epithelial tissues or includes other types of tissues, but it may constitute a novel and interesting field of research.

Conflict of interest

The authors declare no conflict of interest.

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