Potassium Channels Lost During Harvesting of Epithelial Cells are Restored with a Kinetics that Depends on Channel Species

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Key Words

Gene regulation • Kv1.4 • Kv1.6 • Kv1.7 • MaxiK • MDCK cells • Transporting-epithelial-phenotype

Abstract

The polarized distribution of K⁺ channels in MDCK cells is lost upon harvesting and restored upon re-seeding. Using semi-quantitative PCR, in the present work we find that (i) Cells do not “wait” for the normal recycling of membrane proteins to restore their lost channels, but trigger their replacement, suggesting that the membrane has a way of engaging the nucleus. (ii) Replacement channels do not come from an internal reservoir, as it is the case with Na⁺, K⁺-ATPase, but requires a *de novo* synthesis. (iii) Replacement is not an all-or-none response, since mRNA for MaxiK channels increases by 8-fold after re-seeding, but those for Kv1.6 and Kv1.7 are not affected by harvesting/re-seeding. (iv) TEA, charybdotoxin and iberiotoxin fail to trigger the replacement response in mature monolayers,

suggesting that replacement is not due to suppression of channel function. (v) MDCK cells have a typical transporting epithelial phenotype (TEP) consisting of tight junctions (TJs) plus polarity. Although the polarized distribution of K-channels is a prominent attribute of TEP, blocking their function does not perturb the development of TEP, as gauged through the development of TJs, nor level of expression (Western blot) and distribution (confocal microscopy) of occludin, and claudins 1, 3 and 7.

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Introduction

This work is part of an overall attempt to understand why cells express a given type of ion channel, on a given domain of the plasma membrane (polarity), at a certain stage of the cycle, and in a regulated amount. In an effort to answer these questions piecemeal, in earlier works using impaling glass microelectrodes we found that, as

expected, the main specific currents across the plasma membrane of MDCK cells (epithelial line from dog kidney) were carried by K^+ and Cl^- ions [1-3]. Then, using patch clamp methods to independently explore the apical and the basolateral domain, we found that MDCK cells express 4 different species of K^+ channels on the apical domain (of 220, 109, 89 and 31 pS), a fifth on the basolateral (26 pS), plus a single type of Cl^- channel also on the basolateral side. In several hundreds of patches we never found a given channel species on the “wrong” side, indicating that MDCK cells express ion channels in a strictly polarized manner [4-8].

In a later series of works using whole cell clamp techniques, we learned that harvesting the cells with trypsin-EDTA decreases K^+ -conductance (G_K) to a mere 10%, but this parameter is restored within 12-15 hr after plating at confluence, through a process that can be inhibited by actinomycin or cycloheximide [5-8]. However, this does not necessarily means that replenish requires the synthesis of new K^+ channels, as we have previously observed that restoration of Na^+, K^+ -ATPase is also blocked by these inhibitors, yet synthesis of RNA and proteins are needed to produce proteins other than the α and β -subunits of this enzyme [9]. Harvesting and re-seeding also reduces and restores the area of plasma membrane, as gauged through a decrease and a subsequent recovery of electrical capacity. Furthermore, replenish of channels and capacity does not exhibit a straight correlation, suggesting that they proceed through individual mechanism (although, of course, channels are delivered to the plasma already assembled in membrane vesicles). Restitution also requires Ca-activated cell-cell contacts, but this process is not impaired by inhibitors of transcription nor translation, suggesting that Ca^{2+} participates in the delivery of channels to plasma membrane [10], a process that involves phospholipase C, and protein kinase C [5, 10].

These results pose several questions: (i) Do restored channels come from an internal pool as it is the case of Na^+, K^+ -ATPase? (ii) Do they have to be restored by a *de novo* synthesis? (iii) Does the membrane “wait” for the normal recycling of membrane proteins? or (iv) Does the fact that the plasma membrane loses most of its G_K through harvesting, stimulate synthesis and delivery of new channels, indicating that the membrane has a way of telling the nucleus that K^+ channels have been lost, and new synthesis is required? (v) Does restoration occur massively? or (vi) Do cells restore the different ion species with an independent time course? We have previously failed to answer these questions through the use of

specific antibodies against each type of K^+ channel, or Northern blot to measure the mRNA coding for them, because their scarcity in MDCK cells, that had offered the distinct experimental advantage of characterizing them with patch clamp techniques, made it almost impossible to follow this process through immunoblot or fluorescence microscopy. Therefore in the present work, we attempt to answer these questions by resorting primarily to semi-quantitative PCR techniques that, as shown below, have the suitable sensitivity.

Materials and Methods

Cell culture

MDCK cells (derived from dog kidney) were obtained from the American Type Culture Collection (MDCK, CCL-34) and used without cloning. Cell were grown in DMEM, 10% FBS, 100U/ml penicillin and 100 μ g/ml streptomycin (GIBCO, Invitrogen) incubated at 37°C in 5% CO_2 . Plating at confluence in the presence or absence of calcium was as previously described [11]. Potassium channel inhibitors charybdotoxin and iberiotoxin were purchased from Alomone Labs and used a 100 nM final concentration in the media. Tetraethylammonium (Sigma) was used at 2 or 10 mM as indicated in each experiment.

RNA isolation and standard RT-PCR

MDCK cells were plated at confluence and total RNA was obtained after 4, 8, 12, 24, 48 and 72 h. RNA was isolated with the Micro-to-Midi total RNA purification system from Invitrogen. Before cDNA synthesis, the samples were digested with DNase I (Ambion) to eliminate possible genomic DNA contamination. RT-PCR was carried out in two steps, for reverse transcription, Superscript II (Invitrogen) enzyme was used with oligo dT primer and then PCR for the detection of the different mRNA of interest were done from the same cDNA reaction as a starting material. Initially, a PCR reaction was done with general primers for Kv1 family potassium channel that we designed based on an alignment of all the Kv1 family members cDNA sequences reported for human. Forward primer, KV1F: 5'-TTC TTC CTC TTC ATC GGG GTC GTC CT-3' and reverse primer, KV1R: 5'-ATG GCA CAC AGG GAG CCC ACG ATC TT-3'. The 191 bp PCR fragment amplified was cloned with the pCR2.1 TA Cloning Kit (Invitrogen) and independent clones were analyzed by automatic sequencing. (Big Dye v3.1 Kit, Applied Biosystems) and compared against the GenBank with the BLAST program. This strategy allowed us to identify the expression of Kv1.4, Kv1.6 and Kv1.7 voltage dependent potassium channels. After alignment of the three Kv1 channel sequences with the Vector NTI Suite 7.0 Software (Invitrogen) we designed forward primers to amplify specifically each of them, the primers used were: Kv1.4: 5'-GCG GAG GCC GAT GAG CCC ACCA-3'; Kv1.6: 5'-CAG AGG CTG ATG ATG ACG ATT CAC TC-3' and Kv1.7: 5'-GAG GTC GAC CGG GAG GAC TCC CAT-3'. For the amplification of MaxiK potassium channel mRNA we used primers that recognize a region that does not

Fig. 1. Messengers coding for four different ion channels in MDCK cells. (A) Alignment of channels Kv1.4, Kv1.6 and Kv1.7 amplified with the primers KV1F and KV1R. Corresponding sequences were deposited in the GenBank with the accession numbers: DQ462159, DQ462160 and DQ462161 respectively. In spite of shearing a high identity, these show sufficient variation as to enable us to amplify each type separately, using the primers indicated by the underlined segments. (B) Specific PCR amplification of the mRNA of Kv1.6 channel, together with the fragments obtained through restriction with *Rsa* I enzyme (96 and 46 bp) and PCR amplification of the mRNA of Kv1.7 channel and the fragments obtained after restriction with *Dde* I enzyme (102 and 38 bp). The picture also shows the specific PCR amplification of MaxiK, as well as the fragments produced by restriction with enzyme *Dde* I (186 and 64 bp). (C) RT-PCR fragments from the mRNA of β -actin, cyclophilin and ribosomal protein P0. The size of the whole fragments and accession number of the sequences used to design the PCR primers are listed in figure 2C.

present alternative splicing variation equivalent to that used to detect MaxiK in rat in Zarei et al [12]. Sequences of the DsloF and DsloR primers are shown in figure 2C and the sequence of the PCR fragment amplified from MDCK cells was deposited in the GenBank with the accession number DQ462162. Amplification of β -actin, cyclophilin and ribosomal protein P0 were done with PAN-primers designed in our laboratory to amplify these mRNAs sequences from different species (human, mouse, rat and dog). The set of primers used in each transcript are indicated in figure 2C. In some cases, mismatches are present however, specific amplification was observed. In all the PCR reactions a negative control without cDNA was set. Identity of PCR fragments for each mRNA analyzed was confirmed by enzymatic restriction and DNA sequencing.

Semi-quantitative RT-PCR

Relative levels of mRNAs were determined in total RNA from MDCK cells plated at confluence at the time indicated in each experiment. Briefly, 5 μ g of total RNA were used for cDNA synthesis and 1/20 of the reaction used for PCR with the corresponding primer pair to determine the phase of exponential amplification testing different number of cycles and amount of cDNA in the PCR reaction. Linear range of amplification for each mRNA was determined as reported in Vega et al [13] and the conditions used in this work for each mRNA measured are summarized in figure 2C. When potassium channels mRNA levels were quantified, the data were normalized to ribosomal protein P0 mRNA amplified from the same cDNA reaction. Aliquots of 8 μ l out of 50 μ l of PCR reaction were resolved by 2% agarose electrophoresis and images digitalized with the

Fig. 2. Experimental conditions for the semi-quantitative amplification of the mRNA for each channel. (A) Example of amplification for the cDNA of channel Kv1.7, as a function of the number of cycles, showing the intensity of the bands as well as the linearity between the 30th and 36th cycles. On these bases we use 34 cycles of amplification to carry the assay of PCR. (B) Band intensity as a function of the initial amount of cDNA at 34 cycles. The amplification is linear until 2 μl of cDNA added in the reaction. (C): Sequence of the amplification primers, temperature, number of cycles, and initial amount of cDNA determined as above, and used for the semi-quantitative determination of each of the mRNA analyzed.

GeneCam (Syngene) Gel Documentation and Analysis System and the PCR products measured by densitometry with the GeneTool software (Syngene, Cambridge, UK). Quantitative results are shown as means ± SE and the number of repetitions (n) is indicated for each experiment.

Immunofluorescence microscopy

Cells were plated at confluence on coverslips in the presence or absence of potassium channels inhibitors and incubated for 20 h, washed with PBS with Ca²⁺, and then fixed and permeabilized with ice-cold methanol for 10 min.

Immunofluorescence was performed as described in Larre et al [14]. Antibody anti-occludin (Zymed, Cat.No. 33-1500) was used and detected with FITC-labeled goat anti mouse IgG (Zymed, Cat. 81-6511). Samples were mounted in Fluorguard (Bio-Rad) and examined by confocal microscopy SP2 (Leica Microsystems Wetzlar).

Immunoblot

Cell monolayers grown in the indicated conditions were washed three times with ice-cold PBS with Ca²⁺ and samples processed as describe in [14]. The antibodies used were mouse

Fig. 3. Selection of molecular references for the determination of specific mRNA expression. (A): Expression levels of mRNA for β -actin, cyclophilin and P0 ribosomal protein, as a function of time after plating MDCK cells at confluence. (B) Kinetics of mRNA expression of the 3 different proteins, showing the fluctuation of mRNA corresponding to β -actin and cyclophilin, and the stability of the one corresponding to P0 ribosomal protein. Each point is the average of at least 4 different experiments.

anti-occludin, and rabbit anti claudin-1, claudin-3 and claudin-7 all of them from Zymed (cat. 33-1500, 51-9000, 34-1700 and 34-9100 respectively). Mouse anti-actin was a kindly gift of J.M. Hernández (Department of Cell Biology, CINVESTAV) Blot were developed with HRP conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Zymed) and the chemiluminescent detection system ECL Plus (Amersham).

Transepithelial electrical resistance (TER) measurement

The degree of sealing of TJ was assessed by measuring the TER using an EVOM system (World Precision Instruments). Cells were plated at confluence in the presence of drugs on transwells (Costar No. 3415) permeable supports and transferred to the EVOM system for electrical resistance determination. Final values are reported after subtracting resistance of the bathing solution and the empty insert, TER is expressed as $\text{ohm}\cdot\text{cm}^2$ ($\Omega\cdot\text{cm}^2$).

Results

Identifying endogenous K^+ channels in MDCK cells

Identification of the different Kv1 family member of potassium channels expressed in MDCK cells, was based on standard RT-PCR experiments using primers (KV1F, and KV1R) from highly conserved regions in all Kv1 potassium channels. Sequencing the 191 bp PCR fragments obtained allowed us to identify at least three of the potassium channels expressed in MDCK cells as Kv1.4, Kv1.6 and Kv1.7. Alignment of their sequences (fig. 1A) indicates that channels Kv1.4 and Kv1.6 share 73.7% identity, between Kv1.4 and Kv1.7 identity is 73%, and 76% between Kv1.6 and Kv1.7. To the best of our knowledge this is the first time these three channels are found in MDCK dog cells. Although they have a very similar sequence, we were nevertheless able to design specific primers for amplification of Kv1.6 and Kv1.7

independently. As seen in figure 1B, specific PCR amplification of Kv1.6 was confirmed by enzymatic restriction with *Rsa* I (a site which is not present in Kv1.7 PCR fragments) and *Dde* I for Kv1.7 (a site absent from Kv1.6).

Electrophysiological recordings of potassium currents in MDCK in our previous reports indicated that MaxiK, the high-conductance calcium-activated potassium channel, is one of the main endogenous potassium currents in these cells [4]. As expected, we now detect the endogenous expression of MaxiK mRNA in MDCK cells (fig. 1B).

Linear amplification of mRNA coding for specific K^+ channels

Once we identified some of the potassium channels expressed in MDCK cells, in the next step we measured their mRNA levels during the restoration of channels that follows harvesting and re-seeding. In order to have a known amount of mRNA that would serve as reference to normalized the level of mRNA of potassium channels, we measured the expression of three housekeeping genes: β -actin, cyclophilin and ribosomal protein P0, whose RT-PCR amplification fragments are shown in Figure 1C.

Fig. 2 illustrates the method used to measure mRNA as applied to channel Kv1.7. On the basis of fig. 2A we

Fig. 4. The expression of the mRNA for specific ion channels follows an independent kinetics. (A) Expression level of mRNA for potassium channels Kv1.6, Kv1.7 and MaxiK as a function of time after plating. (B) Kinetics of the mRNA level of the three different types of K⁺ channels, as a function of time after plating the MDCK cells at confluence and in the presence of Ca²⁺, showing that each channel species follows its own kinetics. mRNA content for a given channel type in just trypsinated-and-suspended cells was taken as 1.0 (0 h) to normalize and compare contents at different recovery times. Values were also normalized according to the expression of the mRNA for P0 protein. Kv1.6 (●), Kv1.7 (▼) and MaxiK (○); (n = 7). Standard errors for channels Kv1.6 and Kv1.7 are smaller than the symbols used.

picked 34 PCR amplification cycles, and based on the results in fig. 2B we used 1.5 μ l of cDNA for our assays. Fig. 2C summarizes the sequence of the primers used, the amplification conditions, and the amount of cDNA in the PCR reaction, as well as the size of the RT-PCR product obtained for each of the channels species studied. Although these procedures do not yield exact values, it is obvious that the mRNA coding for MaxiK channel is the most abundant, as it requires a smaller number of cycles and a considerable smaller amount of cDNA for its amplification. Figure 2C also provides the values of these parameters for PR P0, the acidic ribosomal protein P0, β -actin and cyclophilin, that were explored for their potential use as reference (fig. 3). Thus Lezama et al [15] have found that β -actin protein remains remarkably constant in subconfluent monolayers of MDCK and during the formation of a confluent polarized monolayer. Yet, in our hands, β -actin mRNA clearly decreases with increasing time at confluence. Cyclophilin instead, provides a clear example of a mRNA for a protein species whose content is considerably sensitive to the functional status of the cell. In turn, P0 mRNA level remains sufficiently constant throughout the harvesting/reseeding process, and was therefore selected to normalize the mRNA levels for the investigated channels.

Kinetics of expression of mRNA for specific channels

Fig. 4A shows examples of the RT-PCR product for each channel type as a function of time after plating at confluence over a period of 3 days. It also includes the band of P0, which was used to normalize the individual values of mRNA. Fig. 4B shows the average of individual measurements. It is clear that the mRNA content for channels Kv1.6 and Kv1.7 are not perturbed by harvesting and reseeding. Production of MaxiK channels receives a distinct enhancement that reaches its peak (more than 8-fold) around the 12th hour. This time course closely matches the recovery of G_K observed in previous work with whole cell clamp techniques [7, 8].

The effect of Ca²⁺

As previously shown [10], MDCK cells recover G_K provided re-plating and incubation are made at confluence, and in the presence of Ca²⁺. This indicates that the process of channel restoration is sensitive to Ca-activated cell-cell contacts. We assayed the content of mRNA for Kv1.6, Kv1.7, MaxiK and P0 at the 20th hour after plating at confluence both, in the presence and the absence of 1.8 mM Ca²⁺ (fig. 5A). Taking the value at 20 hours in the presence of Ca²⁺ as 100% (fig. 5B) it is observed

Fig. 5. The expression of channels mRNA does not depend on the presence of Ca²⁺. (A) Expression level of the mRNA for each channel, in cells that have been plated at confluence for 20 h both, in the presence and in the absence of 1.8 mM Ca²⁺. (B) Densitometric analysis expression of the mRNA each channel species in the absence of Ca²⁺, as percentage of the expression in the presence of this ion.

Fig. 6. K-channel blockers do not affect transcription of MaxiK channel. (A) Expression level of the mRNA for MaxiK in the presence of 100 nM charybdotoxin, 2.0 or 10 mM tetraethyl ammonium. Cells plated at confluence for 3 days were treated for 20 h with these inhibitors. Ribosomal protein P0 was used to normalize mRNA bands of MaxiK. (B) Densitometric analysis. mRNA levels in the presence of inhibitors are expressed as percentage of control level.

that, while harvesting and reseeding drastically induce transcription of MaxiK channels, it may not be affected by the level of extracellular Ca²⁺. Hence, results in fig. 5B may be taken to indicate that newly synthesized channels may be primarily sent to an intracellular reservoir, and functional expression depends on their subsequent transfer to the plasma membrane where Ca²⁺ plays a paramount role. Synthesis of mRNA would be triggered by just the contact between the cell and the support

Might the lack of K-channel-function act as signal to increase transcription?

Since elimination of MaxiK channel by trypsin-EDTA drastically and specifically stimulates transcription of its gene, the obvious suspected membrane-to-nucleus signal is the function of this channel. To test this possibility we inhibited channel function with charybdotoxin [16, 17] or tetraethyl ammonium (TEA). TEA was used at 2.0 mM because at this concentration it inhibits preferentially

Fig. 7. Effect of channel inhibitors on the development of the “transporting epithelial phenotype”. (A–D) Distribution of occludin (green) in monolayers of MDCK that have been plated at confluence for 20h, and whose nuclei were stained (red) with propidium iodide. Since no alteration of the distribution pattern was observed, to avoid crowding only one control transversal sections is shown. (E) Western blot of claudins 1, 3 and 7, as well as occludin. β-actin was used as loading control. Occludin exhibited two different bands. (F) Tight junction formation was gauged through the development of transepithelial electrical resistance (TER). K-channel inhibitors ChTx (100 nM), IbTx (100 nM) and TEA (2.0 mM or 10 mM) were added at zero time and remained present throughout.

MaxiK channels [18], as well as 10 mM because we have observed that at this concentrations it blocks all K-channel currents [4]. Fig. 6 shows that these do not impair the marked increase of MaxiK mRNA observed in fig. 4. As discussed below, this suggests a signaling mechanism of the type of NACOs (Nuclear Adhesion Complexes) [14].

Development of the transporting-epithelial phenotype (TEP) is independent of K channels

As mentioned before, TEP has two main features: TJ and apical/basolateral polarity. The fact that K⁺ and Cl⁻ channels are expressed polarizedly [7, 8], does not necessarily mean that TEP depends on their presence. The relationship between potassium channel function and

development of TEP was investigated through (a) the distribution of occludin, as observed in confocal microscopy (fig. 7A-D). The basic pattern is not affected by the presence of inhibitors of K-channels like charybdotoxin nor iberiotoxin; this one was shown to be the most specific inhibitor available for this channel [17, 19]. (b) The expression level of three different claudins, which are TJ molecules involved in permeation through this structure, and occludin, which are specific markers of TJs (fig. 7E). Occludin shows a heavy band that corresponds to the whole channel, and a lighter one, attributed to a segment of the COO⁻ terminal which is clipped by a caspase [20]. Results in fig. 7E would indicate that ChTx and IbTx inhibit the normal degradation of occludin. (c) The development of transepithelial electrical resistance (TER), which reflects the degree of sealing of the TJ, and therefore is also an indicator of the development of TEP, is not appreciably affected (fig. 7F). The overall conclusion is that, even when the expression of TEP and transcription of MaxiK channels take place simultaneously, they proceed by parallel but basically independent mechanisms.

Discussion

This work is part of an effort to understand why a given cell type expresses a characteristic set of channel species and, if these are lost (e.g. through trypsinization as in the present work) the genome “knows” and sets up a process to replenish the plasma membrane with a new population of the same channels. The study is performed in MDCK cells, which are not excitable, and the small amount of ion channels that they express is below detection with current methods based on sided biotinylation and immunofluorescence. Therefore, in order to study how they modulate their steady state population of channels we resorted to the semi-quantitative RT-PCR approach. This enabled us to find the mRNA of channels Kv1.4, Kv1.6 and Kv1.7, that were not previously reported in this cell type. Presumably, they may be responsible for some of the specific K-conductances found by Ponce et al [7] using patch clamp methods. Reinhardt *et al* [21] did succeed to transfect Kv1.4 in MDCK-F cells; interestingly, they found that in spite of being a transfected channel, it was nevertheless expressed polarizedly, occupying primarily the leading edge. Kv1.6 was found in the collecting duct of the rat kidney [22], and Kv1.7 was localized in the human kidney [23, 24], as well as in cells of heart and skeletal muscle [25].

We observe that, while the expression of some channels (e.g. Kv1.6 and Kv1.7) does not seem to be affected by harvesting/re-seeding, some other (e.g. MaxiK) exhibits drastic changes, as if there were a communication between the plasma membrane and the genome. Therefore we learn that MDCK cells control the different species of channel individually. This is not surprising, as cell from the distal part of the nephron had to adapt to physiological contingencies, such as diet, dehydration, pH, and K⁺, Na⁺ and Cl⁺ variations in plasma, as well as that different stages of the cell cycle [26-31].

Proteins owe their name to the fact that their structure and function are not fixed, but undergo drastic changes in response to minor variations in a mind-boggling number of events. No wonder cells are so “careful” at regulating the type and species of the ions penetrating or leaving a given compartment [32-34]. The case of epithelial cells is even more complex because, besides of requiring ion channels for their own housekeeping, their cytoplasm is continuously flushed by transepithelial fluxes pertaining to the exchange between the organism and the environment. In this respect, the cytoplasm of cells of the distal part of the nephron, i.e. the region of the nephron where MDCK derive from [35], substances penetrate their cytoplasm through one pole and leave at the opposite side of the cell. Furthermore, these transepithelial fluxes are influenced by a host of hormones, as part of the maintenance of the ionic composition of the whole organism.

One of the channel studied was the voltage and Ca-dependent MaxiK, which MDCK cells exclusively express on the apical side [4, 37]. We observe here that the expression of MaxiK is subject to 8-fold time variations (fig. 4). Induction of transcription of MaxiK channels is not affected by Ca-dependent cell-cell contacts which, as shown in a previous study [10] do affect the expression of K channels in the plasma membrane. This suggests that transcription produces the channels, which are primarily sent to internal reservoirs, and Ca²⁺ would be required to incorporate them to the membrane. However, due to the low density of ion channels in MDCK cells mentioned above, does not justify further elaboration on function/expression at this time.

However, the role of this channel cannot be extrapolated to MDCK cells from work with other cell types. Thus, some cells retain MaxiK channels in the endoplasmic reticulum [38]. Rat myometrium has a MaxiK α -subunit variant, called SV1, that is not delivered to the plasma membrane, but accumulates in the vicinity of the nucleus. Interestingly, this is due to a segment of

33 aminoacids, that exhibits a negative dominance preventing surface expression of regular MaxiKs [12, 39]. One may wonder why a cell would spend energy to synthesize an ion channel, to prevent it later on from reaching the plasma membrane. In this respect, it is worth remembering that even intracellular organelles have ion channels, whose population would presumably require independent modulation. Furthermore, some connexins, which are the fundamental proteins of gap junctions, are observed to reside both, at the plasma membrane and within the cell, where they cannot possibly participate in cell-cell communication. Even taking into account that in the present work we are identifying mRNA -not electric currents- we may speculate that when an ion channel (or any other protein, for that matter) accumulates in a situation where it cannot work, it may indicate that it participates in cellular emergencies. Such is the role of ion channels with comparatively low anions/cations selectivity, that are expressed at the surface membrane when cells are subject to sudden changes in the osmolarity of the bathing medium and risk bursting, so their role seems to be the facilitation of a rapidly reduction of osmolite content in the cytoplasm [40].

In a previous work, we investigated the polarized expression of K⁺ channels by transfecting an exogenous *Shaker* one, whose N-terminal was experimentally trimmed to avoid inactivation. Contrary to endogenous channels, the transfected *Shaker* was preferentially -but not exclusively- observed on the basolateral side, presumably for an over-expression that saturated sorting and delivery mechanisms. Nevertheless, through the study of transfected *Shaker* we learned that it is due to an addressing signal of 42 amino acids segment, between amino acids 571 and 613, those results in delivery to a particular domain of the plasma membrane, plus a retention signal involving the last three amino acids of the COOH terminal (threonine, aspartic acid, and valine). This retention is due to a submembrane scaffold of actin, α -actinin, β -tubulin, mLin7 and CASK, that anchors the channel to the cytoskeleton. This polarized expression does not depend on K⁺ permeability, as a mutant of *Shaker* in which a tryptophan in position 434 was replaced by a phenylalanine that completely blocked ion conductance -when expressed in oocytes- did not modify expression or polarity in the membrane of MDCK cells [41]. This is

in keeping with the present observation (fig. 6) that blocking channel function with toxins does not prevent the induction of mRNA. Furthermore, this is a clear indication that channels do not necessarily “communicate” with the genome through their specific role, i.e. through the passage of ions. On the contrary, the chief factor triggering mRNA transcription would be the adhesion of the cells to the substrate.

Therefore an alternative possibility would be a NACo mechanism (Nuclear Adhesion Complexes). NACOs are protein species belonging to the submembrane scaffold of cell-cell and cell-substrate structures, such as TJ, adherent and focal junctions, that depending on the physiological situation at a the contact, depart and travel to the nucleus where they activate or suppress specific genes [14].

Additionally, we observed that although transcriptional induction of MaxiK takes place simultaneously with the transporting epithelial phenotype of MDCK cells there is not any causal relationship between these cellular events (fig. 7).

The fact that we use trypsin-EDTA to elicit a kinetics of replacement of channels, may not indicate that this response would not afford information on physiological situations. Firstly, because, as shown in our previous work, referenced to in the Introduction, the cell responds with the restoration of the same channel species, in the same amount, and the same amount of membrane surface, i.e. a “healing”. Secondly, because cell contacts are crucially altered during cell proliferation, differentiation, and metastasis. This, together with the fact that different species of K⁺ channel are involved in many diseases, would suggest that the present work may contribute to understand and pharmacologically handle clinical situations.

Acknowledgements

We thank the efficient and pleasant technical assistance of Mr. Ángel Marrufo and Elizabeth del Oso. This work was performed with research grants to R.G-V. and M.C. from the CONACYT of México (National Research Council).

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