ELECTRICAL PROPERTIES OF GAP JUNCTION HEMICHANNELS

Interfakultäre Inauguraldissertation
der Philosophisch-naturwissenschaftlichen und
der Medizinischen Fakultät der Universität Bern
zur Erlangung der Doktorwürde

vorgelegt von
Patrick Luzi Bader
von Holderbank SO

Arbeit unter der Leitung von Prof. R. Weingart, Physiologisches Institut, Universität Bern
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von der Philosophisch-naturwissenschaftlichen Fakultät und der Medizinischen Fakultät auf
Antrag der Interfakultären Kommission angenommen.

Bern, den 22.06.2006

Der Dekan der Phil.-nat. Fakultät        Der Dekan der Medizinischen Fakultät

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1. SUMMARY

The classical cell theory, introduced by M.J. Schleiden in 1838, defined cells as 'circumscribed autonomous units' (cf. Loewenstein, 1981). A little over a century later, this paradigm was challenged based on electrophysiological observations. A landmark study by S. Weidmann reported that current is able to flow between cardiac cells, suggesting that cardiac tissue behaves as a functional syncytium (Weidmann, 1952). Shortly thereafter, it was suggested that intercellular membrane channels, so called gap junction channels, are responsible for a direct cell-to-cell communication (Sjöstrand et al., 1958; McNutt and Weinstein, 1973). Later on, it was realized that cells of other tissues, e.g. peripheral nerve (Furshpan and Potter, 1959), smooth muscle (Nagai and Prosser, 1963) or liver (Loewenstein and Kanno, 1967), are also coupled by gap junction channels. Today, we know that gap junctions are present in most tissues of vertebrates and invertebrates where they play a pivotal role in various biological processes, such as growth, development, secretion, wound healing, immune response or impulse propagation. Gap junction channels consist of pairs of hemichannels arranged in series. They provide an aqueous pathway between the cytoplasm of adjacent cells, allowing ions and small molecules to pass from cell to cell.

Over the last decade, rapid progress has been made in understanding the structure, the function, the regulation and the role of gap junctions and their channels. Functional studies were frequently carried out on isolated cell pairs. Recently, experimentalists begun to examine gap junction hemichannels. It is now thought that solitary hemichannels serve functional roles on their own in intact tissues. It has been suggested that they are involved in physiological processes such as signaling between different cells, e.g. between astrocytes in the brain, or horizontal cells and cones in the retina of the eye. No doubt, many more examples will be identified in the near future.
2. INTRODUCTION

2.1. Gap Junction Channels

Gap junctions are specialised membrane structures which are involved in the direct cell-to-cell communication in tissues of vertebrates and invertebrates. They have been studied extensively over the last two decades. This generated a large body of structural and functional information and resulted in a detailed view about the operation of gap junction channels. This chapter attempts to summarise the current knowledge on gap junctions and their channels.

2.1.1. Occurrence and Nomenclature

Immunohistochemical and cytochemical studies demonstrated the occurrence of gap junctions in different tissues of different animal species. It turned out that gap junctions are present in virtually every tissue of the human body, with the exception of blood cells and skeletal muscle cells. Gap junctions are made of integral transmembrane proteins, so called connexins. So far, 20 different connexins have been identified in the human genome and 19 in the mouse genome (Willecke et al., 2002). Many isoforms have species-specific orthologues, bringing the number of naturally occurring connexin variants to over 40 (Beyer and Willecke, 2000). Some isoforms are restricted to specific species, others to specific tissues. The expression of connexin types depends on the kind of tissue and on its state of development and activity. A given connexin can be present in different tissues and more than one type of connexin can be expressed in the same kind of cell. Many cells express more than one type of connexin.

Two nomenclature systems are currently used. The more common one is based on the molecular mass of connexins. For example, connexin45 (Cx45) has a molecular mass of ~45 kDa (cf. Beyer et al., 1988). Species-specific orthologues are designated by a prefix, e.g. mCx45 for mouse, hCx45 for human. To date, the smallest connexin known is Cx25, the largest is Cx58. The less common nomenclature is based on the distinction of 3 families of connexin genes, i.e. α, β and γ (Gimlich et al., 1990). A subscript assigns the order of discovery. For example,
\( \alpha_1 \) stands for Cx43, \( \beta_1 \) for Cx32 and \( \beta_2 \) for Cx26. The former nomenclature will be used in this thesis.

2.1.2. Molecular Structure and Formation of Gap Junctions

This chapter describes the steps involved in the formation of gap junction channels and gap junctions, starting with the smallest unite, the connexin. Each step occurs at a specific site, either inside the cell or in the cell membrane. The developing structure has to travel from site to site. In other words, formation and trafficking of gap junction components are closely linked.

Fig. 1. Basic structure of connexins, the smallest unite of gap junction channels. Connexins are integral membrane proteins that span the membrane four times, thus forming four transmembrane domains (M1–M4), two extracellular loops (EL1, EL2), a cytoplasmic loop (CL) and an intracellular amino (NT) and carboxyl tail (CT).

Connexins (Fig. 1) are synthesised in the endoplasmatic reticulum. The analysis of amino acid sequences revealed that connexins have a common
transmembrane topology. They span the membrane four times, thus forming four \( \alpha \)-helical transmembrane domains which are predominantly hydrophobic (M1 to M4), two extracellular loops (EL1, EL2), a cytoplasmic loop (CL) and a cytoplasmic N- and C-terminal domain (NT, CT). The length of the C-terminal is responsible for most of the differences in molecular mass of the connexins. The overall sequence identity of the different connexin subtypes is approximately 40%, domains M1 to M4 and EL1 and EL2 exhibiting a much greater identity.

Oligomerisation of connexins into connexons (Fig. 2A), the next larger unite of gap a junction, occurs in the Golgi apparatus or in the endoplasmatic reticulum (Yaeger et al., 1998; Martin et al., 2001). Connexons are hexamers of connexins arranged around a central aqueous pore (Unwin and Zampighi, 1980; Unger et al., 1999; Sosinsky et al., 2000). They are also called gap junction hemichannels.

Inserted into the membrane of small vesicles, the connexons migrate along the microtubules to the periphery and fuse into the non-junctional membrane of the cell (Lauf et al., 2002). In this configuration, connexons are free to diffuse laterally within the plasma membrane. Alignment of two connexons from two adjacent cells provokes the formation of a gap junction channel (Fig. 2B). This process involves connexon docking by means of electrostatic forces and opening of the pore of the newly formed channel (Bukauskas and Weingart, 1993b). This takes place at the periphery of an existing gap junction (Gaietta et al., 2002). Older connexins are removed from the centre part of a gap junction and incorporated into vesicles for degradation. Gap junctions are assemblies of closely packed gap junction channels, called gap junctions plaques (Kumar and Gilula, 1996). The size of a plaque ranges from few channels to few thousand channels.

The diverse connexins offer the possibility to form four classes of structurally different channels: i) homomeric-homotypic, consisting of two identical connexons made of the same type of connexin; ii) homomeric-heterotypic, consisting of two different connexons, each made of a different type of connexin; iii) heteromeric-homotypic, consisting of two identical connexons made of more than one kind of connexin; iv) heteromeric-heterotypic, consisting of two different connexons, each made of a different connexin composition.
Fig. 2. (A) Structure of gap junction hemichannel. Arrangement of six connexins grouped around a central pore. (B) Structure of a gap junction channel. Docking of two hemichannels of two adjacent cells results in the formation of a gap junction channel.
2.1.3. Biological Material and Experimental Methods

Gap junction channels can be regarded as barriers for the flow of ions or the diffusion of molecules. Hence, their properties can be studied by means of two different experimental approaches, i.e. measurement of intercellular current to determine the conductance of gap junctions and their channels, or measurement of flux of molecules to assess the permeability of gap junctions and their channels. In my thesis, the focus has been on electrical properties. Hence, the relevant electrophysiological methods and the chosen biological preparations will be described.

Ideally, experiments are performed on cells freshly isolated from tissues. In this way, the extracted data reflect the in vivo situation as close as possible. However, since most wild-type cells express more than one kind of connexin, this renders it difficult to study the electrical properties of gap junctions and gap junction channels made of specific connexins. Cell lines expressing an intrinsic connexin at a negligible level such as HeLa cells, when transfected to express a desired connexin, turned out to be suitable to perform electrophysiological experiments. They are easy to culture and to manipulate during an experiment and are readily available. Alternatively, Xenopus oocytes injected with mRNA coding for various connexins have also been used.

The studies to be described have been carried out on transfected HeLa cells stably expressing mCx45. For experiments, the cells were grown on sterile coverslips placed in culture dishes. After one day or two of growth in the incubator, the cells are ready for use. They form clusters of cells, cell pairs and single cells. Cell pairs are appropriate to study the properties of gap junction channels while single cells are suitable to examine the properties of gap junction hemichannels.

The electrical properties of gap junctions are examined by means of the dual voltage-clamp method in conjunction with whole-cell recording and patch pipettes applied to cell-pair preparations. For this purpose, each cell of a pair is connected via patch pipette to a separate amplifier. The amplifiers enable to control individually the membrane potential of each cell, \( V_{m1} \) and \( V_{m2} \), and thereby to establish desired voltage gradients across the gap junction, \( V_j \), and measure the intercellular current.
elicited, $l_j$. The analysis of the voltage and current signals allows to express the properties of gap junctions or gap junction channels in terms of conductances, i.e. $g_j = l_j / V_j$ or $\gamma_j = l_j / V_j$, respectively. The patch pipettes are pulled from glass capillaries and filled with solution mimicking the ionic composition of the cytoplasm. This approach has been used extensively over the last two decades. As a result, the basic electrical properties, i.e. channel conductance, kinetics and selectivity, of many types of gap junction channels have been characterized (cf. Harris, 2001). Likewise, the electrical properties of gap junction hemichannels are examined by means of the single voltage-clamp method in conjunction with whole-cell recording applied to single cell preparations. In this case, the analysis furnishes the conductances $g_{hc} = l_m / V_m$ or $\gamma_{hc} = l_m / V_m$, respectively.

### 2.1.4. Properties of Gap Junction Channels

Early studies on tightly coupled cell pairs suggested that vertebrate gap junction channels exhibit a constant conductance (e.g. White et al., 1983; Weingart, 1986). Later on, when less tightly coupled cell pairs were examined, it was found that $l_j$ remained constant at small $V_j$, but decreased with time at large $V_j$, indicating voltage gating (e.g. Veenstra, 1990; Bukauskas and Weingart, 1993a). This behavior was previously missed because of series resistance complications arising from an unfavorable ratio $R_{gj} / R_{pip}$ ($R_{gj}$: gap junction resistance; $R_{pip}$: pipette resistance; cf. Van Rijen et al., 1998). Related studies established another kind of gating, i.e. chemical gating, mediated by intracellular ions such as Ca$^{2+}$ or H$^+$, and lipophilic agents such as n-alkanols (e.g. Turin and Warner, 1977; Spray et al., 1982; Niggli et al., 1989). Incomplete uncoupling of cell pairs induced by lipophilic agents, e.g. n-heptanol or anesthetics, revealed single channels currents (e.g. Veenstra and DeHaan, 1986; Burt and Spray, 1988; Rook et al., 1988; Rüdisüli & Weingart, 1989). Introducing the induced cell-pair preparation, it became possible to determine unambiguously the single channel conductances (Bukauskas and Weingart, 1993b; Bukauskas et al., 1995a). These studies revealed that gap junction channels exhibit several conductance states. The two gating mechanisms will be explained next.
2.1.4.1. Voltage Gating

Gap junction channels of vertebrates are sensitive to the transjunctional voltage, $V_j$, those of invertebrates are sensitive not only to $V_j$, but also to $V_m$, the membrane potential (e.g. Bukauskas and Weingart, 1994). This chapter will elaborate on $V_j$-gating only. For the sake of simplicity, the case of homomeric-homotypic channels is considered. Based on several studies performed on cell pairs expressing different kinds of connexins, the following picture of gap junction channel operation has emerged (cf. Harris, 2001). The results obtained from experiments on induced cell pairs turned out to be most valuable (Bukauskas and Weingart, 1994; Bukauskas et al., 1995a).

![Diagram](image.png)

**Fig. 3.** Independent gating of connexons of a gap junction channel. (A) Resting situation. No voltage drop occurs over the gap junction channel ($V_1 = V_2 = -60$ mV, $V_j = 0$ mV) and both connexons have their lumen opened. (B) Depolarization of the upper cell ($V_1 = 0$ mV, $V_2 = -60$ mV, $V_j = -60$ mV) results in gating of the connexon with the respective gating polarity. (C) Mirror of situation B ($V_1 = -60$ mV, $V_2 = 0$ mV, $V_j = +60$ mV).

Each channel has a voltage-sensitive gating mechanism which consists of two gates in series responsive to $V_j$ (Fig. 3). Each connexon accommodates one gate located near the cytoplasmic region of the channel pore. The two gates are acting independently. Hence, at a given voltage only one of the gates is responding, depending on the polarity of its voltage sensor. Each connexon contributes six subgates attributable to the six connexin subunits. Their operation leads to fast transitions ($< 1-2$ ms) between the channel main state (i.e. fully open channel) and
the residual state (i.e. partially closed channel) and 5 substates in between. The channels do not go into a closed state (i.e. fully closed channel). The transitions between the main state and the residual state are frequent, those involving substates are rare. The probability of a channel to be in the open state, $P_o$, is controlled by $V_j$.

At $V_j = 0$ mV, the channel is most of the time in the open state (i.e. $P_o \approx 1$), at large $V_j$ it is mainly in the residual state (i.e. $P_o \approx 0$), and at intermediate $V_j$ it is in the main state, a substate or the residual state (i.e. $1 > P_o > 0$). The decay of gap junction currents with time (inactivation of $I_j$) at large $V_j$ is determined primarily by $P_o$ and, to a lesser degree, by the occurrence of substates. The residual state may be regarded as ground state of electrical coupling. The conductances of both the main state and the residual state are $V_j$-sensitive. The conductances of a hemichannel are voltage sensitive as well (e.g. Trexler et al., 1996; Valiunas and Weingart, 2000). This behavior has been anticipated from the properties of heterotypic gap junction channels (Bukauskas et al., 1995b).

For illustration, Fig. 4 shows single channel current records obtained from an induced pair of transfected HeLa cells expressing mCx40 (from Bukauskas et al., 1995a). At the beginning of the experiment, two separate cells were brought into physical contact with each other. This enabled opposed connexons to dock at their cytoplasmic aspect and form gap junction channels. The currents were recorded immediately after the formation of the first operational channel. $V_1$ and $V_2$ correspond to the membrane potential of cell 1 and cell 2, respectively, while the traces $I_1$ represent the gap junction current, $I_j$. Initially, both cells were clamped to the same membrane potential ($V_1 = V_2 = -50$ mV). Thereafter, hyperpolarization of cell 2 to -125 mV led to a transjunctional potential $V_j$ of -75 mV ($V_2 - V_1$) provoking a current flow through the gap junction channel ($I_1$). The signal $I_1$ revealed fast transitions between different current levels attributable to the channel main state ($I_{j,\text{main state}}$), the residual state ($I_{j,\text{residual state}}$; long dashes) and several substates ($I_{j,\text{substate}}$; short dashes) in between. Such current records yielded the following mean values for the single channel conductances: $\gamma_{j,\text{main state}} = 198$ pS; $\gamma_{j,\text{residual state}} = 36$ pS.

The conductance of single gap junction channels varies considerably among different types of channels (cf. Harris, 2001). The value of $\gamma_{j,\text{main state}}$ can be as small
as 15 pS (Cx36; Teubner et al., 2000) or as large as 375 pS (Cx37; Kumari et al., 2000). Likewise, $\gamma_{j,\text{residual state}}$ can be 3.8-fold (Cx32) to 6.6-fold (Cx30) smaller than the respective $\gamma_{j,\text{main state}}$.

**Fig. 4.** Conductance states of a gap junction. The upper panel illustrates the voltage protocols applied to cell one and cell two ($V_1$ and $V_2$). Cell one was constantly clamped to -50 mV ($V_1 = -50$ mV). Cell two was repetitively hyperpolarized from -50 mV ($V_2 = -50$ mV, $V_j = 0$ mV) to -125 mV (200ms, $V_2 = -125$ mV, $V_j = -75$ mV). The lower panel shows three associated current signals measured in cell one ($I_1$) exhibiting rapid flickering between different conductance levels. They involve the main state (maximal current), the residual state (---), several substates (- - -) and the closed state (-----). (From Bukauskas et al., 1995a)
Figure 5A shows multichannel current records obtained from a pair of HeLa cells expressing mCx40 (from Bukauskas et al., 1995a). Again, both cells were clamped to the same holding potential ($V_1 = V_2 = -50$ mV). Depolarization of cell 1 to 25 mV ($V_j = -75$ mV) gave rise to a junctional current $I_j$ with a time-dependent decay not reaching the zero level (trace $I_2$, left-hand side). Hyperpolarization of cell 1 to -125 mV ($V_j = 75$ mV) led to a similar $I_j$ of opposite polarity (trace $I_2$, right-hand side).

Figure 5B shows the results collected from 7 cell pairs. $V_j$ was varied over a broad range of voltages. The analysis involved the following steps: i) determination of the amplitude of $I_j$ at the beginning ($I_{j,\text{inst}}$; inst = instantaneous) and end ($I_{j,\text{ss}}$; ss = steady state) of each $V_j$ pulse; ii) calculation of the respective conductances $g_{j,\text{inst}} = I_{j,\text{inst}} / V_j$ and $g_{j,\text{ss}} = I_{j,\text{ss}} / V_j$; iii) normalization of the $g_{j,\text{ss}}$ values with respect to $g_{j,\text{inst}}$; iv) plot of the $g_{j,\text{ss}}$ data versus $V_j$. The resulting graph is symmetrical; $g_{j,\text{ss}}$ is maximal at small $V_j$ and decreases at 25 mV < |$V_j$| < 75 mV, without dropping off to zero conductance, even at the largest $V_j$ gradient. The single channel properties offer the following explanation for the incomplete decay of $g_{j,\text{ss}}$ at large $V_j$: the channels do not close completely, they remain in a residual conductance state.

The smooth curve in Fig. 5B represents the fit of data to the Boltzmann equation: $g_j = \{(1 - g_{j,\text{min}}) / [1 + \exp(A(V_j - V_{j,0}))]\} + g_{j,\text{min}}$ ($V_{j,0}$: $V_j$ where $g_{j,\text{ss}}$ is half-maximal; $g_{j,\text{min}}$: minimal conductance at large $V_j$; $A$: steepness of $g_{j,\text{ss}}/g_{j,\text{inst}}$ changes). It allows to characterize gap junctions in biophysical terms. The Boltzmann parameters obtained are: $V_{j,0} = 47$ mV, $g_{j,\text{min}} = 0.25$, $A = 0.235$.

Different gap junctions exhibit different Boltzmann parameters (cf. Harris, 2001). For example, Cx45 is strongly voltage sensitive ($V_{j,0} = 31$ mV, $V_{j,\text{min}} = 0.07$, $A = 0.08$; Barrio et al., 1997), and Cx26 is weakly voltage sensitive ($V_{j,0} = 85$ mV; $g_{j,\text{min}} = 0.27$; $A = 0.14$; Barrio et al., 1991).
Fig. 5. Dependency of gap junction conductance, $g_j$, on voltage gradient across the gap junction, $V_j$. (A) $V_1$ and $V_2$: pulse protocol applied to cell one and cell two giving rise to a $V_j$ of -75 mV ($V_1 = 25$ mV, $V_2 = -50$ mV; left-hand side) and 75 mV ($V_1 = -125$ mV, $V_2 = -50$ mV; right-hand side). $I_2$: associated current measured in cell two exhibiting a time-dependent decay. (B) Relationship between $g_j$ at steady state, $g_{j,ss}$, and $V_j$. Each symbol corresponds to a single determination, $n = 7$. The smooth curve represents the best fit to the Boltzmann equation. For parameters see text. (From Bukauskas et al., 1995a)
2.1.4.2. Chemical Gating

Besides voltage-gating, gap junction channels also have a chemical-gating mechanism. It is responsive to intracellular ions, such as Ca^{2+} and H^+, or lipophilic agents, such as long-chain alkanols (e.g. heptanol) and fatty acids (cf. Harris, 2001; Peracchia, 2004). Characteristic of chemical gating are the channel closed state and the *slow transitions* (tens of ms) between a channel open state, i.e. the main state, a substate or the residual state, and the channel closed state. There is evidence that different channels exhibit different chemical sensitivities (cf. Harris, 2001). As a rule, the effects of these agents are reversible.

Figure 6 illustrates an example of chemical gating. It shows current records obtained from a pair of neonatal rat heart cells (Valiunas et al., 1997). They predominantly express Cx43, the major cardiac gap junction protein. Superfusion with solution containing an uncoupling agent, 75 µM SKF-525A, provoked complete inhibition of $I_j$ (not shown). During washout of the agent, it was possible to follow the sequential re-opening of previously closed channels. To detect the reappearance of channel activity, $V_j$ was maintained at -60 mV ($V_1 = 12$ mV, $V_2 = -48$ mV). The paired current records ($I_1$ and $I_2$) in the upper panel show the first channel re-opening (left-hand side), followed by few fast transitions between the main state and residual state (dashed lines) and a re-closure (right-hand side). The lower panel repeats the early and late episode of $I_1$ and $I_2$ at an expanded time scale. It demonstrates that the channel re-opening and re-closure involved slow transitions (~25 ms) between the fully open state and the completely closed state. In between, the channel flickered between the fully open state and the residual state, reflecting voltage gating. Slow transitions between the residual state and the closed state do also occur.

Such interventions are frequently used to impair intercellular coupling and study single channel properties. Lipophilic agents affect gap junction channels in a non-specific manner. It is generally believed that they act via insertion into the lipid bilayer of the cell membrane (Burt et al., 1991).

Chemical gating induced by H^+ and Ca^{2+} is most important *in vivo*. A decrease in pH_i typically produces a decrease in junctional conductance. The sensitivity to H^+ varies among cell types and is related to the connexin involved (cf. Harris, 2001;
Peracchia, 2004). One line of evidence, based on connexin truncation experiments, suggests that low pH facilitates the interaction between the CT and CL domain via protonation of amino acid residues, causing the channel to close (Morley et al., 1997). Another line of evidence, based on intracellular pH buffer studies, suggests a modulatory role of protonated aminosulfonates via occupation of a binding site on the CT domain (Bevans and Harris, 1999).

**Fig. 6.** Recruitment of a gap junction channel previously blocked by exposure to 75 µmol/L SKF-525A. The upper panel shows a current record ($I_1$ and $I_2$) of a newly recruited channel. After re-opening, the channel flickered between the open state and the residual state. Later on, it closed completely again. The lower panel shows segments of $I_1$ and $I_2$ at expanded time scale. Transitions between the closed state and main state were slow ($\approx$ 25 ms); those between the main state and the residual state were fast ($<2$ ms). Solid lines: zero current level; dashed lines: residual current level. $V_1$ = 12 mV, $V_2$ = -48 mV, $V_j$ = 60 mV. $\gamma_{j,\text{main state}}$ = 68 pS, $\gamma_{j,\text{residual state}}$ = 13 pS. (From Valiunas et al., 1997)
With regard to Ca\(^{2+}\), this divalent cation has long been known to interfere with gap junctions. Already before the identification of individual channels, it was reported that Ca\(^{2+}\) is critically involved in the process of healing over of cardiac tissue (Délèze, 1970). However, despite extensive efforts ever since, the mode of action of Ca\(^{2+}\) remains still unclear. Conceivably, it may directly act on the gap junction proteins, concomitantly with pH\(_i\) changes, or indirectly via cytoplasmic intermediates, such as calmodulin, and thus close the channels. Moreover, there is uncertainty about the critical threshold concentration of Ca\(^{2+}\). Some studies reported that [Ca\(^{2+}\)]\(_i\) as low as 150 nM to few µM is sufficient (Dahl et al., 1980; Noma et al., 1987; Rose et al., 1987; Peracchia et al., 1990; Lazrak et al., 1993; Enkvist et al., 1994). Other studies estimated a critical concentration of as high as 40 to 400 µM (Oliveira-Castro et al., 1971; Spray et al., 1982; Firek et al., 1995). It appears that the [Ca\(^{2+}\)]\(_i\) required to block gap junction channels varies among connexins and cell types (Peracchia, 2004).

**2.1.5. Functional Role of Gap Junction Channels**

Gap junctions establish a direct connection between the cytoplasm of adjacent cells and hence allow the transfer of ions or small molecules. The former permits the flow of intercellular current which enables action potential propagation in the heart (Kléber and Rudy, 2004). The latter allows the diffusion of second messengers and metabolites with a molecular mass up to 1 or 2 kDa and plays a role in all connexin expressing tissues (cf. Harris, 2001). Gap junctions play a pivotal role in a wide range of biological processes, such as embryogenesis, development, differentiation, proliferation, hormone secretion, wound healing, lens tissue homeostasis, immune functions, or impulse propagation in cardiac muscle, smooth muscle or neuronal networks.

Because of its importance, the role of gap junctions in cardiac physiology will be further considered. The contraction of the myocardium is crucial for the blood flow in the body. It results from the concerted contraction of its cardiomyocytes. Each cardiomyocyte is activated by an action potential. The action potential is generated in the sinus node. From there, it propagates throughout the heart following a standard
pattern. In this way, each cell receives a trigger signal at the appropriate time. It is generally accepted that the conduction of an action potential occurs via local circuit currents. They represent closed loops that involve four elements: 1) the excitatory inward current carried by Na\(^+\) and/or Ca\(^{2+}\); 2) the intracellular longitudinal resistance, \(r_i\) (two resistances in series, the cytoplasmic resistance \(r_c\) and the gap junction resistance \(r_{gj}\); 3) the membrane capacitance, 4) the extracellular longitudinal resistance. In essence, the conduction velocity of an action potential is determined by these four elements. From the functional point of view, elements 1), 2) and 4) are of particular interest because they are variable. The role of 1) is well established. Over the last decade or so, it turned out that 2) can be modified under certain conditions.

Under pathophysiological conditions, such as myocardial infarction, the properties of gap junctions are impaired. Ischemia provokes disturbances of the impulse propagation via impairment of gap junction channels. This results from an elevation of [Ca\(^{2+}\)] (via inhibition of the Na\(^+\)/K\(^+\)-Pump and the Na\(^+\)/Ca\(^{2+}\)-exchanger), a decrease in pH (via disturbance of the metabolism) and a release of arachidonic acid from the lipid bilayer of cell membranes. Closure of the channels at the edge of an infarcted area, i.e. between injured cells and healthy cells, delimit the infarcted area. In this way, areas of damaged tissue remain limited such that the heart may still keep its pump function, although at reduced efficiency (Severs, 1999). This phenomenon is known as healing over.

### 2.2. Gap Junction Hemichannels

Initially, gap junction hemichannels have been studied to better understand the properties of gap junction channels. However, there is recent evidence suggesting that solitary hemichannels may have functional roles beyond serving as precursors of gap junctions (Ebihara, 2003; John et al., 2003). As a result, hemichannels with their properties and functions mutated to a research area on its own.
2.2.1. Evidence for Hemichannels

Early structural evidence for the existence of hemichannels in the plasma membrane came from freeze-fracture studies (Ryerse et al., 1984). A decade later, hemichannels embedded in cell membranes were identified by means of Western blot analysis of biotinylated cell surface proteins (Musil and Goodenough, 1993). After another decade, fluorescence microscopy was used to monitor gap junction formation from its precursors (Gaietta et al., 2002; Lauf et al., 2002; Segretain and Falk, 2004). These studies demonstrated that connexons dwell in the non-junctional membrane prior to docking with connexons of a neighbouring cell. This finding was consistent with previous immunocytochemical studies suggesting the presence of Cx43 in non-contacting regions of cell membranes (el Aoumari et al., 1991; Rook et al., 1992). Cx43 in non-contacting regions was also detected in mammalian cells expressing Cx43 linked to green fluorescence protein, GFP (John et al., 1999).

Functional evidence for operational hemichannels came from electrophysiological and diffusional experiments. Initially, a novel current was detected in horizontal cells of catfish and skate retina (DeFries and Schwartz, 1992; Malchow et al., 1993). This current had properties appropriate for gap junction hemichannels. It was apparent in solution with reduced [Ca$^{2+}$]o, was sensitive to voltage and regulated by pH. Moreover, the cells became permeable to Lucifer yellow (mol. wt. 457), a dye commonly used to identify gap junction channels. Similar results emerged from experiments performed on injected Xenopus oocytes expressing rat Cx46 (Paul et al., 1991; Ebihara and Steiner, 1993) and transfected cells expressing various kinds of vertebrate connexins (cf. Sáez et al., 2003; cf. John et al., 2003).

2.2.2. Experimental Methods

Like gap junction channels, hemichannels can be characterised in terms of electrical or diffusional properties. This chapter elaborates on the methods appropriate for the former.

The electrophysiological properties of hemichannels are best examined with a single voltage-clamp method. Single cells are connected via a patch pipette to an
amplifier. The amplifier allows to control the membrane potential, \(V_m\), and measure the associated transmembrane current, \(I_m\). The resulting conductances are \(g_{hc} = I_m / V_m\) or \(\gamma_{hc} = I_m / V_m\) for multichannel or single channel preparations, respectively.

Cells are generally patched in the presence of physiological \([Ca^{2+}]_o\), i.e. \(\sim 2\) mM. In this situation, hemichannels are usually closed. Reducing \([Ca^{2+}]_o\) results in the opening of hemichannels. The decrease in \([Ca^{2+}]_o\) is achieved by superfusion with solution containing EGTA to buffer the free \(Ca^{2+}\) at a desired low level. In the experiments to be presented, solution changes were made via a conventional system for bulk solution changes (exchange time: \(\sim 1-2\) min) or via a pipette positioned close to a cell for local solution changes (exchange time: \(\sim 0.3\) s).

![Equivalent circuit of a cell membrane consisting of a capacitance \(C_m\), background ion channels \(R_x\) and gap junction channels \(R_{hc}\). A membrane current \(I_m\) may consist of a current arising from the capacitance of the cell membrane \(I_c\), a current flowing through background channels \(I_x\) and of a current flowing through hemichannels \(I_{hc}\). In order to measure currents exclusively carried by hemichannels, the capacitive current \(I_c\) has to be minimized electronically and the background currents \(I_x\) have to be blocked pharmacologically.](image)

**Fig. 7.** Equivalent circuit of a cell membrane consisting of a capacitance \((C_m)\), background ion channels \((R_x)\) and gap junction channels \((R_{hc})\). A membrane current \((I_m)\) may consist of a current arising from the capacitance of the cell membrane \((I_c)\), a current flowing through background channels \((I_x)\) and of a current flowing through hemichannels \((I_{hc})\). In order to measure currents exclusively carried by hemichannels, the capacitive current \((I_c)\) has to be minimized electronically and the background currents \((I_x)\) have to be blocked pharmacologically.
In order to gain reliable hemichannel currents, the following aspects have to be considered:
Hemichannels embedded in the cell membrane are arranged in parallel with intrinsic ion channels, such as $K^+$ or $Cl^-$ channels giving rise to a background current, $I_x$, and with the capacitance of the cell membrane leading to $I_c$ (Fig. 7).
i) Hence, the membrane current, $I_m$, corresponds to the sum of the individual current components. In order to study hemichannel properties, the hemichannel current, $I_{hc}$, has to be isolated pharmacologically by blocking the background channels.

ii) The capacitance of the pipette ($C_{pip}$) and the cell membrane ($C_m$; Fig. 7) slows down the response time of the voltage clamp and hence must be kept small. To reduce the former, in the experiments to be presented the tip of the pipette was coated with a silicon elastomer (Silgard). The remaining capacitance as well as $C_m$ were then reduced by means of the capacity-compensation circuit of the amplifier.

iii) The seal resistance between the pipette tip and the membrane has to be in the gigaohm range to minimize current leaks between the inside of the cell and the bath. This is essential when working with HeLa cells. They are small and thus have a high input resistance.

### 2.2.3. Properties of Hemichannels

Since gap junction channels represent series arrangements of two hemichannels, one might think that gap junction channel properties can be predicted from hemichannel properties. However, it turns out that this is not always the case. This chapter summarizes the current knowledge on hemichannel properties.

The chemical gating mechanism of hemichannels will be first explained. At physiological $[Ca^{2+}]_o$ (2 mM), hemichannels are usually closed. A number of studies reported that lowering $[Ca^{2+}]_o$ provokes hemichannels to open (Paul et al., 1991; Trexler et al., 1996; Ebihara, 1996; Pfahnl et al., 1997; Ebihara et al., 1999; Pfahnl and Dahl, 1999; Beahm and Hall, 2002). There is evidence that the associated current transitions are slow (tens of ms; Valiunas and Weingart, 2000). The critical concentration at which hemichannels open is still under debate. It may be different for different types of hemichannels. One study used a $[Ca^{2+}]_o$ in the low nM range to
study Cx30, Cx46 and Cx50 hemichannels (Valiunas and Weingart, 2000). Another study examined Cx45 hemichannels in saline with no added Ca$^{2+}$ (Valiunas, 2002). Due to contamination, [Ca$^{2+}$]$_{o}$ might have been as large as 10 µM. Yet other studies reported Cx46 and Cx50 hemichannels to open already at millimolar [Ca$^{2+}$]$_{o}$ (Beahm and Hall, 2002; Ebihara et al., 2003). Like gap junction channels, hemichannels are not only affected by Ca$^{2+}$, but also by protons (Sáez et al., 2005).

Some hemichannels can also been made to open when the level of the intracellular ATP$^{2-}$ is decreased. This has been shown for Cx43 in cardiomyocytes and in transfected HEK239 cells (John et al., 1999; Kondo et al., 2000). The interventions used included exposure to FCCP or iodoacetate. These agents inhibit the oxidative or glycolytic metabolism, thereby lowering [ATP$^{2-}$].

Inhibitors of gap junction channels, such as glycyrrhetinic acid or trivalent cations (La$^{3+}$ or Gd$^{3+}$), are also able to reversibly close hemichannels (Contreras et al., 2002).

Once hemichannels are open, their electrophysiological properties can be studied. Several investigations indicated that they are sensitive to the transmembrane voltage, $V_{m}$. Depolarization or hyperpolarization either activate or deactivate the channels, depending on the polarity of the gating mechanism. A change of $V_{m}$ leads to transitions involving the main state, the residual state and several substates (Trexler et al., 1996; Valiunas and Weingart, 2000; Valiunas, 2002). The current records indicate that the transitions are fast, i.e they occur within 1-2 ms (Trexler et al., 1996; Valiunas and Weingart, 2000). The conductance of a hemichannel in the open state is approximately twice that of a gap junction channel as predicted from the series arrangement of two connexons. So far, the basic properties of the following homomeric hemichannels have been characterized: Cx34, Cx35, Cx45, Cx46 and Cx50 (Trexler et al., 2000; Valiunas and Weingart, 2000; Valiunas, 2002; Contreras et al., 2003).
2.2.4. Functional Role of Hemichannels

The finding that hemichannels are able to open under certain conditions brings up the following question: do these channels play a role physiologically and/or pathophysiologically? Evidence for a functional role emerges from a correlation between hemichannel activity and cytoplasmic ATP\(^2\)-established for tissues of the heart and the central nervous system. This chapter summarizes the current knowledge in this research area.

Half a decade ago, two studies proposed that hemichannels are involved in pathophysiological conditions (John et al., 1999; Kondo et al., 2000). They showed an opening of Cx43 hemichannels upon a reduction of [ATP\(^2\)]. The experiments were performed on dispersed cardiomyocytes and transfected HEK239 cells expressing Cx43. Exposure to FCCP or iodoacetate, blockers of oxidative or glycolytic metabolism, respectively, lead to a decrease in [ATP\(^2\)]. This provoked the activation of a non-selective membrane current and the uptake of gap junction-permeable hydrophilic dyes, both suggested to be mediated by hemichannels. A functional implication of this observation may be as follows. During myocardial infarction, local ischemia leads to ATP\(^2\)-depletion and subsequent opening of hemichannels. The latter allows an influx of Ca\(^{2+}\) which forces gap junction channels to close, thereby separating injured cells from healthy cells. This mechanism may induce the process of healing over (see section 2.1.4.2. Chemical gating).

Cx43 hemichannels in rat and mouse cortical astrocytes were also found to open upon metabolic insults (Contreras et al., 2002). In these experiments, ATP\(^2\)-depletion was followed by permeabilization of the cell membrane to Lucifer yellow and ethidium bromide, dyes diffusing through gap junction channels. Opening of hemichannels may initiate a collapse of the electrochemical and metabolic gradient across the cell membrane of dying astrocytes. These authors also examined the effect of ATP\(^2\)-depletion on gap junctions. It revealed a reduction but no block of intercellular communication. They suggested that healthy neighbouring astrocytes might protect dying cells via open gap junction channels (Contreras et al, 2004). Alternatively, the gap junction channels may allow the transfer of necrotic or apoptotic signals from cells with open hemichannels to unaffected neighboring cells.
Further evidence for a functional role of hemichannels arises from experiments on the intercellular communication of astrocytes (Cotrina et al., 1998; Stout et al., 2002). Astrocytes are capable of widespread intercellular communication via propagated increase in \([\text{Ca}^{2+}]_i\). Cell-to-cell propagation of these \(\text{Ca}^{2+}\) waves seems to be mediated by ATP\(^2\) as an extracellular messenger (Zhu et al., 1991; Charles et al., 1992; Naus et al., 1992; Cotrina et al., 1998; Hofer et al., 1998; Guthrie et al., 1999; Cotrina et al., 2000; Stout et al., 2002). There is evidence that ATP\(^2\) diffuses from the intracellular to the extracellular space via open Cx43 hemichannels. Interestingly, \(\text{Ca}^{2+}\) waves of astrocytes have been shown to modulate synaptic signalling between neurons, suggesting a role in synaptic plasticity (Araque et al., 1998; Kang et al., 1998; Parpura et al., 2000). They have also been shown to modulate the response of retinal neurons to light stimulation (Newman et al., 1998). Astrocyte \(\text{Ca}^{2+}\) waves have also been implicated in migraine headache and spread of seizures (Charles, 1998).

A functional role of hemichannels was also found in horizontal cells of the outer retina (Kamermans et al., 2001). An essential feature of the first synapse in the retina is a negative feedback pathway from horizontal cells to cones, involving Cx26 hemichannels in the horizontal cells. Blocking of these hemichannels abolished the feedback-mediated responses.

### 2.3. Aim of Present Study

This study consists of two closely related subprojects. The goal of the first subproject has been to determine the basic electrophysiological properties of Cx45 hemichannels at the macroscopic current level. Cx45 was chosen because of its broad distribution in the heart (prominently expressed in specialised tissues, i.e. SA-node, AV-node, bundle branches and PF; marginally present in atrium and ventricle). It is vital for the propagation of the electrical impulse in the heart. For example, the slow impulse propagation in the AV-node appears to be correlated with the low expression level of Cx45 in this specialized tissue. Hence, dysfunction of Cx45 renders this tissue susceptible to disturbances of impulse propagation. Moreover, Cx45 was found to be up-regulated under pathophysiological conditions leading to
heart failure (Yamada et al., 2003). Overexpression of Cx45 results in greater susceptibility to tachyarrhythmias and sudden cardiac death (Betsuyaku et al., 2006). The aim of this subproject was to augment the knowledge on the basic properties of gap junction hemichannels and hence to better understand the properties of gap junction channels. Of particular interest were the voltage dependence of the conductance and the kinetics, as well as the polarity of voltage gating. Moreover, it was driven by the growing evidence that hemichannels exert functional roles on their own under physiological and pathophysiological conditions, e.g. in myocardium and the central nervous system.

The goal of the second subproject has been to optimise the experimental conditions to quantitatively determine the electrophysiological properties of Cx45 hemichannels at the macroscopic current level. It emerged from initial observations of the first project (see above). A problem was the variability of the putative hemichannel currents. Systematic alterations of the experimental conditions (growth conditions, ionic composition and osmolarity of solutions, use of channel blockers) eventually let to satisfying results. Critical was the unexpected interference of a current carried by volume-regulated anion channels (VRAC) with properties resembling those of hemichannel currents. Distinct experimental protocols enabled to distinguish the currents and determine their biophysical (conductance, kinetics) and pharmacological properties. The results obtained raise questions concerning the interpretation of previously published work on hemichannel currents. Interestingly, the properties of VRAC and hemichannels suggest their sequential involvement in an autocrine/paracrine signalling pathway activated by osmotic stress under physiological (e.g. proliferation) and pathophysiological conditions (e.g. ischemia). A recent study suggests a functional interaction between a chloride channel (cystic fibrosis transmembrane conductance regulator, CFTR) and Cx45 gap junction channels (Kotsias and Peracchia, 2005). Dysfunction of CFTR channels causes the genetic disease cystic fibrosis. In this study, performed on Xenopus oocytes transfected with CFTR and Cx45, activation of CFTR channels caused an increase in junctional conductance and a reduction in transjunctional voltage sensitivity of
Cx45. Therefore, pathogenesis of cystic fibrosis may not depend entirely on altered transmembrane $\text{Cl}^-$ transport.
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4. PUBLISHED WORK

4.1. Conductive and kinetic properties of connexin45 hemichannels expressed in transfected HeLa cells
Conductive and Kinetic Properties of Connexin45 Hemichannels Expressed in Transfected HeLa Cells

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Received: 12 November 2003/Revised: 2 April 2004

Abstract. Human HeLa cells transfected with mouse connexin Cx45 were used to examine the conductive and kinetic properties of Cx45 hemichannels. The experiments were carried out on single cells using a voltage-clamp method. Lowering the \([Ca^{2+}])_0 revealed an extra current. Its sensitivity to extracellular Ca\(^{2+}\) and gap junction channel blockers (18β-glycyrrhetinic acid, palmitoleic acid, heptanol), and its absence in non-transfected HeLa cells suggested that it is carried by Cx45 hemichannels. The conductive and kinetic properties of this current, \(I_{hc}\), were determined adopting a biphasic pulse protocol. \(I_{hc}\) activated at positive \(V_m\) and deactivated partially at negative \(V_m\). The analysis of the instantaneous \(I_{hc}\) yielded a linear function \(g_{hc,\text{inst}} = f(V_m)\) with a hint of a negative slope (\(g_{hc,\text{inst}}\): instantaneous conductance). The analysis of the steady-state \(I_{hc}\) revealed a sigmoidal function \(g_{hc,\omega} = f(V_m)\) best described with the Boltzmann equation: \(V_{m,0} = -1.08\) mV, \(g_{hc,\text{inst}} = 0.08 (g_{hc,\omega}, \text{ steady-state conductance}; V_{m,0}: V_m\) at which \(g_{hc,\omega}\) is half-maximally activated; \(g_{hc,\text{inst}}: \text{ minimal conductance}; \text{ major charge carriers: K}^+\) and Cl\(^-\)). The \(g_{hc}\) was minimal at negative \(V_m\) and maximal at positive \(V_m\). This suggests that Cx45 connexons integrated in gap junction channels are gating with negative voltage. \(I_{hc}\) deactivated exponentially with time, giving rise to single time constants, \(\tau_d\). The function \(\tau_d = f(V_m)\) was exponential and increased with positive \(V_m\) (\(\tau_d = 7.6\) s at \(V_m = 0\) mV). The activation of \(I_{hc}\) followed the sum of two exponentials giving rise to the time constants, \(\tau_{a1}\) and \(\tau_{a2}\). The function \(\tau_{a1} = f(V_m)\) and \(\tau_{a2} = f(V_m)\) were bell-shaped and yielded a maximum of \(\approx 0.6\) s at \(V_m \approx -20\) mV and \(\approx 4.9\) s at \(V_m \approx 15\) mV, respectively. Neither \(\tau_{a1} = f(V_m)\) nor \(\tau_{a2} = f(V_m)\) coincided with \(\tau_d = f(V_m)\). These findings conflict with the notion that activation and deactivation follow a simple reversible reaction scheme governed by first-order voltage-dependent processes.

Key words: Gap junction hemichannel — Connexin45 — Conductance — Kinetics — Electrophysiology — Heart

Introduction

Gap junction channels provide a direct pathway for the exchange of mediators of intercellular signaling. They consist of two hemichannels or connexons arranged in series. Each connexon contains 6 subunits or connexins forming an aqueous pore sufficiently large to accommodate ions or small molecules. Connexins are integral membrane proteins that span the membrane four times, thus forming four transmembrane domains (M1–M4), two extracellular loops (E1, E2), a cytoplasmic loop (CL) and an intracellular amino (NT) and carboxyl tail (CT). So far, 19 different connexins have been identified in the mouse genome and 20 in the human genome (cf. Willecke et al., 2002). Connexins are transcripts of a multi-gene family.

The trafficking and assembly of connexins into gap junction channels have been investigated extensively (Martin et al., 2001). There is evidence for the coexistence of two parallel pathways. The classical route involves oligomerization of connexins into hexamers in the Golgi apparatus and subsequent transport to the plasma membrane; an alternative route involves initiation of oligomerization in the ER and transport to the plasma membrane bypassing the Golgi. Both concepts suggest that hemichannels are already present in the plasma membrane of single cells. This is consistent with the results from experiments examining the de novo formation of gap junction channels (Bukauskas and Weingart, 1994).

Cell-pair preparations have been widely used to study the electrical properties of gap junctions and

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gap junction channels (cf. Bruzzone White & Paul 1996). The parameters gained from these studies allowed the design of a mathematical model that describes the operation of the channels (Vogel & Weingart, 1998). More recently, it has been reported that gap junction hemichannels can be examined in cellular preparations using isolated primary cells (De Vries & Schwartz, 1992; Kondo et al., 2000), injected oocytes (Ebihara, Berthoud & Beyer, 1995; Trexler et al., 1996) or transfected cell lines (Li et al., 1996; Valiunas & Weingart, 2000; Valiunas, 2002).

The connexin Cx45 is prominently expressed in the cardiovascular system, primarily in the SA-node, the AV-node and the bundle branches (Gros & Jongsma, 1996; Severs et al., 2001). It has also been found in other tissues such as smooth muscle and neurons (cf. Willecke et al., 2002). The electrical properties of Cx45 gap junction channels have been studied by several laboratories. They exhibit a low unitary conductance and a high voltage sensitivity (Barrio et al., 1997; Van Veen et al., 2000; Elenes et al., 2001). More recently, Cx45 hemichannels have been used to explore the electrical and diffusional behavior (Valiunas, 2002).

The aim of this study was to determine the conductive and kinetic properties of Cx45 hemichannels at the multichannel level. To this end, we have used transfected HeLa cells expressing mouse Cx45 (mCx45 or mouse connexin 26; molecular mass or Greek letter nomenclature, respectively; Butterweck et al., 1994; Elfgang et al., 1995). We have determined the instantaneous and steady-state TF relationship and elucidated the voltage dependence of In deactivation and activation. The data gained are relevant for the heart during tachycardia prevailing both during physiological and pathophysiological conditions. Preliminary data have been published elsewhere in abstract form (Bader & Weingart, 2003).

Materials and Methods

CELLS AND CULTURE CONDITIONS

Transfected human HeLa cells expressing mouse connexin45, mCx45 (Butterweck et al., 1994; Elfgang et al., 1995), and non-transfected HeLa cells were grown in Dulbecco’s medium (DMEM) containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 U/ml penicillin (2212 Seromed; Falkola, Basel, Switzerland). Transfected cells were selected using 0.5-1 μg puromycin (P-7255; Sigma). For experiments, cells were harvested in DMEM with 10% FCS (2×0.2 ml of ~1×10³ cells/ml) and seeded onto sterile glass coverslips placed in multi-well culture dishes and used within 24 h after plating.

SOLUTIONS

Experiments were carried out in K⁺-rich solution containing normal Ca²⁺ (in mM): KCl 140, NaCl 4, CaCl₂ 2, MgCl₂ 1, HEPES 5 (pH = 7.4), glucose 5, pyruvate 2, CaCl₂ 2, BaCl₂ 1, TEA⁺-Cl⁻ 2; or K⁺-rich solution (Continuing a reduced [Ca²⁺] (in mM): CaCl₂ 1, EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetra-acetic acid) 10 (free Ca²⁺: 7.6 mM corresponding to a pCa = 8.1). Patch pipettes were filled with regular pipette solution (in mM): KCl 140, NaCl 4, CaCl₂ 1, MgCl₂ 1, Mg-ATP 3, HEPES 5 (pH = 7.2), EGTA 5 (free Ca²⁺: 43 mM, corresponding to pCa = 7.4), CsCl 2, BaCl₂ 1, TEA-Cl 2, Ba²⁺, Ca²⁺, Cs⁺ and TEA⁺ served to block the K⁺ channels. Mibefradil (20 μm; a gift from Roche Pharma, Basel, Switzerland) was added to the extracellular solutions to block the volume-regulated Cl⁻ channels (Nilius et al., 1998; Bader & Weingart, unpublished results). 18-glycerophosphoric acid (G-5803; Sigma) and (pamitioleic acid (P-9417; Sigma) dissolved in DMSO and hexane (10 mM stock solutions), respectively, before dilution in K⁺-rich solution.

ELECTRICAL MEASUREMENTS

Glass coverslips with adherent cells were transferred to a chamber superfused with KCl-containing solution at room temperature (22-26°C). The chamber was mounted on the stage of an inverted microscope equipped with phase-contrast optics (Diaphot-TMD, Nikon; Nippon Kogaku, Tokyo, Japan). Patch pipettes were pulled from glass capillaries (GC150F-10; Harvard Apparatus, Edenbridge, UK) using a horizontal puller (DMZ-Universal; Zeitz Instruments, Munich, Germany). The tip of the pipettes was coated with a silicon elastomer to reduce the capacitance (Sylgard 184; Dow Corning, Wiesbaden, Germany). When filled with solution, the pipettes had DC resistances of 2-6 MΩ. Experiments were carried out on single cells using a single voltage-clamp method (Valiunas & Weingart, 2000). After selection of a cell, a gigahm-seal was produced and the conditions for whole-cell recording were established. The patch pipette was fixed in a pipette holder mounted on a micromanipulator (MP-258; Sutter Instrument Novato, USA) and connected to an amplifier (EPC 7; HEKA Elektronik, Darmstadt, Germany). The method permitted control of the membrane potential Vm and measurement of the associated current, Im.

SIGNAL RECORDING AND ANALYSIS

For analysis, unless otherwise stated, the signals were filtered at 1 kHz (8-pole Bessel filter) and digitized at 3.33 kHz with an A-D converter (ITC-16, Instrument, Fort Washington, N.Y., USA). Data acquisition and analysis were done with the software Pulse/PulseFit (HEKA Elektronik). Curve fitting and statistical analysis were performed with SigmaPlot and SigmaStat, respectively (Jandel Scientific, Erkrath, Germany). The results are presented as means ± SEM.

Results

RECRUITMENT OF HEMICHANNELS

Figure 1 illustrates the procedure used to open putative hemichannels. A HeLa cell expressing mCx45 was patched in the presence of K⁺-rich solution with normal Ca²⁺ (2 mM). After establishing the whole-cell configuration, the membrane potential, Vm, was measured, being close to 0 mV, as expected for [K⁺]o = [K⁺]i. After activation of the voltage-clamp, a biphasic voltage pulse was applied every
30 s. Starting from a holding potential \( V_h = 0 \) mV, \( V_m \) was first depolarized to 30 mV for 10 s and then hyperpolarized to \(-40\) mV for 5 s (Fig. 1A). At time \( t = 0 \) min, low-Ca\(^{2+}\) solution (7.6 mM) was washed in. Figure 1B shows 2 superimposed current traces, \( I_m \), recorded during control and 3 min after the intervention. During control, \( I_m \) showed a small outward component of constant amplitude, followed by a small inward component of constant amplitude. In the presence of low [Ca\(^{2+}\)], the depolarizing step was accompanied by a large outward component that increased with time, while the hyperpolarizing step was associated with a large inward component that decreased with time to a level different from the reference level.

Figure 1C illustrates the change of \( I_m \) during wash-in of low-Ca\(^{2+}\) solution. For this purpose, the amplitudes of the following current components were determined and plotted as a function of time: \( I_{m,\text{max}} \) (maximal); \( \Delta I_{m,\text{inst}} \) (inst: instantaneous; O) and \( I_{m,\text{ss}} \) (ss: steady state; ●), prevailing at the end of the depolarizing pulse, the beginning of the hyperpolarizing pulse and the end of the hyperpolarizing pulse, respectively. During wash-in of low-Ca\(^{2+}\) solution, \( I_{m,\text{max}} \) and \( I_{m,\text{ss}} \) increased from nearly zero to a substantial outward and inward current, respectively, while \( I_{m,\text{ss}} \) increased only slightly. The similar time course of \( I_{m,\text{max}} \), \( I_{m,\text{inst}} \) and \( I_{m,\text{ss}} \) is consistent with a sequential recruitment of hemichannel currents. At hyperpolarizing steps, \( I_{m,\text{ss}} \) was always much smaller than \( I_{m,\text{inst}} \). This is in agreement with a transition of hemichannels from a completely open state to a partially closed state. The latter is reminiscent of the residual state of gap junction channels. Upon return to Ca\(^{2+}\)-containing solution, the extra current components vanished (data not shown). This suggests that the hemichannels that had opened previously closed again. In fact, the small current components observed during the control period disappeared virtually completely. This means that the channels responsible for the initial background current were blocked and/or downregulated. Hence, an interference from these channels was negligible. In a given cell, the Ca\(^{2+}\)-sensitive extra current could be recruited repetitively and showed consistent properties.

To ascertain that the extra \( I_m \) is mediated by Cx45 hemichannels, the following control experiments were performed (data not shown). Examining Cx45-HeLa cells exposed to low-Ca\(^{2+}\) solution, we found that the extra \( I_m \) was reversibly blocked by the gap junction blockers 182-glycyrhrhetic acid (40 μM), palmitoleic acid (40 μM) or heptanol (3 mM) (cf. Harris, 2001). Moreover, non-transfected HeLa cells exposed to low-Ca\(^{2+}\) solution failed to generate an extra \( I_m \). These observations support the view that the Ca\(^{2+}\)-sensitive extra current is carried by Cx45 hemichannels. Hence, it was called hemichannel current, \( I_{hc} \).

**Fig. 1.** Recruitment of hemichannel currents. (A) Biphasic voltage pulse to elicit currents carried by hemichannels. Starting from 0 mV, \( V_m \) was depolarized to 30 mV for 10 s and then hyperpolarized to \(-40\) mV for 5 s. Dotted line, \( V_m = 0 \) mV. (B) Superimposed current traces \( I_m \) recorded in control solution (pCa, 2.7) and after wash-in of low-Ca\(^{2+}\) solution (pCa, 8.1) for 3 min. The intervention was started at time \( t = 0 \) min. The extra current components are attributable to hemichannels. The amplitude of \( I_m \) at the end of the depolarizing pulse corresponds to \( I_{m,\text{max}} \) (maximal); the amplitude of \( I_m \) at the beginning and end of the hyperpolarizing pulse correspond to \( I_{m,\text{ss}} \) (inst: instantaneous) and \( I_{m,\text{ss}} \) (ss: steady state), respectively. The dotted line corresponds to the zero-current level. (C) Plot of current components as a function of time after exposure to low-Ca\(^{2+}\) solution. (A) \( I_{m,\text{max}} \) (O), \( I_{m,\text{inst}} \) (●), \( I_{m,\text{ss}} \).

**VOLTAGE DEPENDENCE OF HEMICHANNEL CURRENTS**

Cells with operational hemichannels were then used to examine the voltage dependence of \( I_{hc} \). This involved the biphasic pulse protocol consisting of a conditioning pulse followed by a test pulse. The conditioning pulse of constant amplitude activated the current, the test pulse of variable amplitude served to study the properties of \( I_{hc} \). Preliminary experiments revealed that a conditioning pulse to 30 mV for 10 or 20 s fully activated \( I_{hc} \). The subsequent test pulse was varied in amplitude and duration. It covered the voltage range between \(-50\) and
60 mV, using increments of 5, 10 or 20 mV and lasted 7 to 20 s, depending on the time required for \( I_{hc} \) to reach a new steady-state level. The biphasic pulses were repeated at intervals long enough for \( I_{hc} \) to recover, i.e., 10 to 30 s, depending on \( V_m \) of the test pulse. \( V_h \) was again set to 0 mV.

Figure 2.4 shows the voltage protocol applied and Fig. 2B, the associated currents recorded. The conditioning pulse elicited an outward current. After a sudden rise, it increased gradually with time, approaching a steady state. The initial level reflects the current through the channels already in the main state plus the channels in the residual state at \( V_m = 0 \) mV; the quasi steady-state level reflects the current through all the channels in the main state. The subsequent test pulse yielded \( I_{hc} \) signals whose direction and contour was dependent on \( V_m \). Hyperpolarization of \( V_m \) gave rise to inward currents with an instantaneous peak. \( I_{hc,inst} \) followed by a decrease to a steady-state level, \( I_{hc,ss} \) (\( V_m = -50, -30, -15, -5 \) mV). The latter was different from the holding current, suggesting an incomplete deactivation. The more negative \( V_m \), the larger was the amplitude of \( I_{hc,inst} \) and the faster the time course of decay. Depolarization of \( V_m \) led to outward currents with virtually no change in time (\( V_m = 15 \) mV) or a small time-dependent increase (\( V_m = 40 \) mV). The more positive \( V_m \), the larger was the amplitude of \( I_{hc,inst} \) and \( I_{hc,ss} \).

For analysis, the amplitudes of \( I_{hc,inst} \) and \( I_{hc,ss} \) were determined for each test pulse. To account for spontaneous changes during an experimental run, the amplitude of \( I_{hc,max} \) was used as a reference. The values \( I_{hc} \) corrected in this way were plotted as a function of \( V_m \), as shown in Fig. 3. Each symbol corresponds to a single determination. The function \( I_{hc,inst} = f(V_m) \) (○) yielded a nearly constant slope. At the largest values of \( V_m \), there was a tendency to deviate from linearity, i.e., the data points were bending towards the x-axis. In contrast, the function \( I_{hc,ss} = f(V_m) \) (●) showed a prominent break around \( V_m = 0 \) mV. The data points at negative \( V_m \) were significantly smaller than those at positive \( V_m \). While the latter yielded a quasi-linear relationship, the former did not.

The values \( I_{hc,inst} \) and \( I_{hc,ss} \) were then used to calculate the conductances \( g_{hc,inst} \) and \( g_{hc,ss} \), respectively. After normalization, the conductances gained from 8 cells were sampled, averaged and plotted as a function of \( V_m \). In order to normalize the \( g_{hc,inst} \) data, a value extrapolated to \( V_m = 0 \) mV was used as reference. It was obtained by averaging the values of \( g_{hc,inst} \) at nearby voltages, i.e., ± 5 mV. In order to normalize the \( g_{hc,ss} \) data, the values of \( g_{hc,ss} \) from each cell were expressed as a fraction of \( g_{hc,inst} \) prevailing during the same pulse. Figure 4.4 shows the plots of the function \( g_{hc,inst} = f(V_m) \). The solid line represents the result of a linear regression analysis. Over the voltage range examined, \( g_{hc,inst} \) showed a linear relationship with a moderate negative slope (zero \( V_m \) intercept: 0.98; slope = −0.01 mV). The correlation between \( g_{hc,inst} \) and \( V_m \) was significant statistically (regression coefficient \( r = 0.6; P < 0.025 \)).

As shown in Fig. 4B, the function \( g_{hc,ss} = f(V_m) \) was sigmoidal with a maximum at positive \( V_m \) and a minimum at negative \( V_m \). The latter was distinctly different from zero, consistent with the view that most channels altered from the main state to the residual state, while few remained in the main state (Valinivas, 2002). The transition of \( g_{hc,ss} \) from maximum to minimum occurred between about −40 and 40 mV. The smooth curve represents the best fit of data to the Boltzmann equation

\[
g_{hc,ss} = \frac{g_{hc,max} - g_{hc,min}}{1 + e^{-A(V_m-V_{m,0})}} + g_{hc,min} \tag{1}
\]

where \( g_{hc,max} \) and \( g_{hc,min} \) are the maximal and minimal conductances at large positive and negative \( V_m \), respectively. \( V_{m,0} \) corresponds to \( V_m \) for which \( g_{hc,ss} \) is half-maximally activated. \( A \) is a constant expressing gating charge \( zq(kT)^{-1} \); \( z \) = unitary positive charges \( q \) moving through the electric field applied; \( k \) = Boltzmann constant; \( T \) = temperature in Kelvin; cf. Harris, Spray & Bennett, 1981). The analysis yielded the following values: \( V_{m,0} = -1.08 \) mV; \( g_{hc,max} = 1.04 \), \( g_{hc,min} = 0.08 \); \( z = 4.0 \). The function \( g_{hc,ss} = f(V_m) \) reflects the gating behavior of Cx45 channels and hence is relevant for the operation of Cx45 gap
junction channels (cf. Elenes et al., 2001). The value of \( g_{hc} \) at \( V_m = 60 \) mV did not fit unequivocally into the set of data points. Hence, it was omitted from the curve-fitting procedure. Conceivably, it reflects an unspecified effect of Cx45 hemichannels and/or the contribution of an unknown current system.

Deactivation of Hemichannel Currents

The current signals elicited by the bipolar-pulse protocol were also used to study the kinetics of \( I_{hc} \) deactivation (Fig. 5A). Figure 5B shows selected current records obtained at a test potential of \(-20\), \(-40\), and \(-50\) mV (from top to bottom). They indicate that \( I_{hc} \) deactivated faster when \( V_m \) was made more negative (see also Fig. 2). For analysis, the \( I_{hc} \) signals were subjected to a least-square curve-fitting procedure. It turned out that \( I_{hc} \) deactivation proceeded with a monoeponential time course. The smooth curves superimposed on the current traces correspond to the best fit of data to the equation

\[
I_{hc}(t) = (I_{hc,\text{inst}} - I_{hc,SS}) \cdot e^{-\frac{t}{\tau_d}} + I_{hc,SS}
\]

where \( \tau_d \) is the time constant of \( I_{hc} \) deactivation. The analysis of the records yielded the following values: \( \tau_d = 6.7, 1.5 \) and \( 0.9 \) s.

Figure 6 summarizes the \( \tau_d \) data gathered from 8 cells. The values of \( \tau_d \) were determined from individual \( I_{hc} \) records, averaged and plotted as a function of \( V_m \). The graph includes data from current signals with a monotonic change in time. This was the case for voltages ranging from \(-50\) to \(5\) mV. At \( V_m > 5\) mV, the records showed outward currents with no detectable decay or an inconsistent behavior, such as a small sustained or transient increase. Hence they were excluded from the analysis. Over the voltage range considered, \( \tau_d \) increased progressively as \( V_m \) was made more positive. The smooth curve corresponds to the best fit of data to the exponential

\[
\tau_d = \tau_{d,0} \cdot e(V_m/V_c),
\]

where \( \tau_{d,0} \) is the zero \( V_m \) intercept and \( V_c \) the decay constant. The analysis yielded the following values: \( \tau_{d,0} = 7.6 \) s, and \( V_c = 19.7 \) mV.

Activation of \( I_{hc} \)

Figure 7A illustrates the pulse protocol adopted to explore the activation of \( I_{hc} \). Starting from a \( V_h \) of
Figure 7B shows a continuous current record obtained at a return potential of 15 mV. This initiated the process of $I_{hc}$ activation. On the one hand, this is visible as a time-dependent increase of the outward current (continuous trace interrupted by short current spikes) indicating a progressive activation of the channels. On the other hand, this is discernible as a pulse-to-pulse increase of the inward current spikes elicited by the short test pulses (envelope method). The spike amplitude eventually reached at steady state reflects the number of channels in the main state plus the number of channels in the residual state at $V_m = 15$ mV. Figure 7C illustrates the analysis of the current spikes elicited by the test pulses. The procedure utilized involved the following steps. To determine the initial amplitude of $I_{hc}$, the current segment over the first millisecond was discarded because of the limited response time of the recording set-up. The remaining segment was extrapolated linearly to the time $t = 0$ s, which indicated the beginning of the activation process. The values of $I_{hc,inst}$ obtained were then plotted as a function of time (●). The graph shows that $I_{hc}$ increased with time. The smooth curve represents the best fit of data to the sum of two exponentials:

$$I_{hc}(t) = I_{hc}(0) + C_1 \left[ 1 - e^{-\left(t / \tau_{a1}\right)} \right] + C_2 \left[ 1 - e^{-\left(t / \tau_{a2}\right)} \right].$$

(4)

$I_{hc}(0)$ is the current at time $t = 0$ s and corresponds to $I_{hc,inst}$ associated with the hyperpolarizing pulse; $\tau_{a1}$ and $\tau_{a2}$ are the time constants of activation. The parameters $C_1$ and $C_2$ signify the respective amplitudes of $I_{hc}$ at steady state and correspond to the
Hence, these values are comparable to those gained with the envelope method.

In an independent series of experiments, the two procedures of determining $\tau_a$ were applied sequentially rather than simultaneously. A comparison of the results revealed no systematic differences. For example, at a return potential of 30 mV, the respective time constants were as follows (envelope method/curve fitting procedure): $\tau_{a1} = 0.46/0.53$ s and $\tau_{a2} = 4.4/4.8$ s. The similarity of data gained with the two procedures suggests that the envelope method, in conjunction with the criteria chosen, is reliable to study the kinetics of $I_{he}$ activation. Since it allows more accurate measurements at $V_m$ close to the reversal potential, it has been used for further experiments.

To elucidate the voltage sensitivity of $I_{he}$ activation, a series of experiments was performed stepping the return potential to different levels, i.e., $-40, -30, -20, -15, -10, -5, 5, 10, 15, 20, 30$ and 50 mV. At each return potential, the values of $I_{he,inst}$ elicited by the test pulses were determined, normalized with respect to $I_{he,inst}$ associated with the hyperpolarizing pre-pulse, and the respective conductances calculated. The normalized values of $g_{he,inst}$ were then sampled, averaged and plotted as a function of time. The graph in Fig. 8 illustrates the results from 4 complete experiments. For clarity, it includes the data for selected voltages only, i.e., $V_m = -30, -15, -5, 5, 15$ and 30 mV (from bottom to top). It indicates that $g_{he}$ activated in a voltage-dependent manner. The activation was faster and less complete at more negative $V_m$, and slower and more complete at more positive $V_m$. At voltages negative to $-15$ mV, the data were best fitted by a simple exponential giving rise to $\tau_{a1}$, and at more positive voltages, by the sum of two exponentials giving rise to $\tau_{a1}$ and $\tau_{a2}$. The smooth curves represent the best result of the curve fitting.

The time constants derived from the data presented in Fig. 8 and others from the same experiments were then used to establish the voltage dependence of $\tau_{a1}$ and $\tau_{a2}$. Figure 9A shows the resulting plots of the functions $\tau_{a1} = f(V_m)$ (○) and $\tau_{a2} = f(V_m)$ (◼), which characterize the fast and slow process of activation, respectively. It indicates that the two functions differ with respect to values and voltage sensitivity. On the one hand, $\tau_{a1}$ was maximal at $V_m \approx -20$ mV and declined more strongly at positive voltage than at negative voltage. On the other hand, $\tau_{a2}$ was maximal at $V_m \approx 15$ mV and decayed more prominently at negative voltage than at positive voltage. Furthermore, over the voltage range that yielded useful results, the values of $\tau_{a1}$ were smaller than those of $\tau_{a2}$. Interestingly, a comparison of Figs. 6 and 9A indicates that there is no correspondence between the $\tau_{a}$ data and the $\tau_{a1}$ or $\tau_{a2}$ data.

The parameters $C_1$ and $C_2$ gained from the analysis were used to establish the relationships...
Fig. 8. Voltage dependence of $g_{ac}$ activation. The activation of $g_{ac}$ was explored with the envelope method (for details, see text). The data collected were sampled, averaged and plotted as a function of time. The results depicted were obtained at $V_{in} = -30, -15, -5, 5, 15$ and $30$ mV (from bottom to top). The symbols correspond to mean values ± 1 SEM ($n = 4$). To emphasize the early phase of activation, the data at $V_{in} = 5$ mV are represented by the open circles. The smooth curves correspond to the best fit of data to the sum of two exponentials.

$C_1 = f(V_m)$ (●) and $C_2 = f(V_m)$ (○), which characterize the contribution of the fast and slow activation process at steady state, respectively. Figure 9B shows the resulting plots. The smooth curves represent the best fit of data to the Boltzmann equation

$$C = \frac{C_{\text{max}}}{1 + e^{\frac{V_m - V_{0,0}}{z}}},$$

(5)

$C_{\text{max }}$ is the maximal $C$ at positive $V_m$ and $V_{0,0}$ corresponds to the voltage at which $C$ is half-maximal (for further explanations, see Eq. 1). The analysis yielded the following parameters: fast process, $V_{0,0} = 3.2$ mV, $C_{1,\text{max }} = 0.50$, $z = 2.0$; slow process, $V_{0,0} = -9.6$ mV, $C_{2,\text{max }} = 0.37$, $z = 6.8$. Considering the values of $C_{1,\text{max}}$ and $C_{2,\text{max}}$, the maximal contribution of the fast and slow process amounts to 58 and 42%, respectively. A comparison of the two curves indicates that the two functions also differ with respect to values and voltage sensitivity. The fast process (●) was less voltage sensitive, operated over a broader $V_m$ range and contributed a larger maximal amount. The slow process (○) was more voltage sensitive, operated over a narrower $V_m$ range and contributed a smaller maximal amount.

**Discussion**

The results indicate that mouse Cx45 hemichannels expressed in human HeLa cells open up under appropriate experimental conditions, i.e., low [Ca$^{2+}$]o and a depolarized $V_m$. This is consistent with a recent study of mCx45 (m: mouse) and cCx45 (c: chicken) hemichannels expressed in HeLa and RIN cells, respectively (Valiumas, 2002). The recruitment of operational hemichannels occurred in a [Ca$^{2+}$]o-dependent manner (Bader, Weingart, Egger; unpublished observations) and was reversible. A concentration of 10 nm was adequate. It opened a maximal number of channels, preserved the basic properties of the cell membrane and kept the cells alive.

**Voltage Dependence of Hemichannel Currents**

A biphasic pulse protocol was used to examine the properties of Cx45 hemichannel currents, $I_{ac}$. The constant conditioning pulse served to activate the channels and the variable test pulse, to establish a
driving force of different amplitude and either polarity. This enabled us to study the conductive and kinetic properties of \( I_{\text{bac}} \). The analysis of \( I_{\text{bac,inst}} \), the current at the onset of the test pulse, yielded a linear function \( g_{\text{bac,inst}} = f(V_m) \) with a negative slope significantly different from zero (see Fig. 4.4). Assuming that \( g_{\text{bac,inst}} \) does not involve channel gating (Vogel & Weingart, 1998), \( g_{\text{bac,inst}} = f(V_m) \) is expected to reflect the sum of the conductance of single hemichannels in the main open state, \( g_{\text{bac,inst,main}} \), i.e., \( g_{\text{bac,inst}} = n \cdot g_{\text{bac,main}} \) (\( n \): number of channels). Hence, the function \( g_{\text{bac,inst}} = f(V_m) \) is expected to resemble the relationship \( g_{\text{bac,main}} \) at \( f(V_m) \). Indeed, it has been reported that \( g_{\text{bac,main}} \) exhibits a negative slope giving rise to larger values at negative \( V_m \) and smaller ones at positive \( V_m \) (Valunias, 2002).

Are there other factors that may influence the function \( g_{\text{bac,inst}} = f(V_m) \)? HeLa cells exhibit two types of inward rectifying K\(^+\) channels, one being voltage-dependent and Ca\(^2+\)-insensitive (Sa"uve, Roy & Payet, 1983), the other one, voltage-independent and Ca\(^2+\)-sensitive (Sa"uve et al., 1986; Diaz & Sepulveda, 1995), and a volume-activated Ca\(^2+\) current (Sae"gharani et al., 2001). Interference from these channels was minimized by adding channel blockers to the solutions (see Materials and Methods; see also Valunias & Weingart, 2000). However, their contribution cannot be excluded completely. Another possibility is the limiting frequency response of the recording setup. However, the precautions taken to minimize this problem render it unlikely: use of low-resistance pipettes, coating of the pipette tips, use of a capacity-compensation circuit and curve-fitting with back extrapolation to determine \( g_{\text{bac,inst}} \).

The analysis of \( I_{\text{bac,ss}} \), the current at the end of the test pulse, revealed a sigmoidal function \( g_{\text{bac,ss}} = f(V_m) \) with a maximum at positive \( V_m \) and a minimum at negative \( V_m \) (see Fig. 4.4). The best fit of data to the Boltzmann equation yielded the following values: \( V_{m,0} = -1 \text{ mV}, g_{\text{bac,max}} = 1.04, g_{\text{bac,min}} = 0.08, \xi = 4.0 \). Several conclusions emerge from this relationship. (i) The decay of \( g_{\text{bac,ss}} \) at negative \( V_m \) suggests that gap junction channels made of mCx45 hemichannels are gating with a negative polarity. (ii) The decay of \( g_{\text{bac,ss}} \) from maximum to minimum occurred over a voltage range of about 80 mV. This is consistent with the behavior of Cx45 gap junction channels if one assumes that half of the transjunctional voltage drops across each hemichannel (cf. Vogel & Weingart, 1998). (iii) The \( g_{\text{bac,ss}} \) did not decline to zero at large negative \( V_m \). According to the general concept of gap junction channel behavior (see, e.g., Valunias et al., 1999), this suggests that the Cx45 hemichannels switched from the main state to the residual state. However, this concept may need modification, since it has been found that few Cx45 hemichannels remain in the open position at large negative \( V_m \) (Valunias, 2002). (iv) The function \( g_{\text{bac,ss}} = f(V_m) \) crossed the zero-voltage axis at \( g_{\text{bac,ss}} = 0.60 \), indicating that a significant fraction of hemichannels dwelled in the residual state under this condition. This suggests that a similar fraction of Cx45 gap junction channels is in the residual state at \( V_m = 0 \text{ mV} \) as well. Indeed, it has been reported that not all of these channels are open at \( V_m = 0 \text{ mV} \) (Moreno et al., 1995; see also Elenes et al., 2001). However, the fraction of open channels is considerably larger than predicted from our data on Cx45 hemichannels. Hence, the voltage sensitivity of the hemichannels may be altered by the docking process (cf. Valunias & Weingart, 2000). (v) The \( g_{\text{bac,ss}} \) reached a maximum at \( V_m = 40 \text{ mV} \) but moderately declined at more positive voltages. This may be due to a contribution from other channels (see above). (vi) The function \( g_{\text{bac,ss}} = f(V_m) \) reached a minimum at \( V_m = -40 \text{ mV} \). This and the sensitivity to extracellular Ca\(^{2+}\) (see Recruitment of Hemichannels) render it unlikely that solitary Cx45 hemichannels are open in cardiac myocytes under physiological conditions (\( V_m = -60 \) to \(-90 \text{ mV} \)).

A similar relationship \( g_{\text{bac,ss}} = f(V_m) \) has been reported recently for mCx45 hemichannels \( (V_{m,0} = 11.1 \text{ mV}, g_{\text{bac,max}} = 2.34, g_{\text{bac,min}} = 0.21, \xi = 1.7; \) Valunias, 2002). However, this study revealed a more positive \( V_{m,0} \) and a constant \( g_{\text{bac,ss}} \) at large positive \( V_m \). These differences may reflect the different ionic solutions used (K\(^+\) aspartate versus KCl). The ratio \( g_{\text{bac,ss}}/g_{\text{bac,ss}} \) was comparable in their and our studies (0.09 versus 0.08), while \( \xi \) was smaller in theirs (1.7 versus 4.0). The high voltage sensitivity of mCx45 hemichannels is consistent with that seen in mCx45 gap junction channels (Barrio et al., 1997; Elenes et al., 2001).

**Kinetics of Hemichannel Currents**

The current signals gained with the bipolar pulse protocol revealed that \( I_{\text{bac}} \) deactivation is governed by \( V_m \). The time-dependent decay of \( I_{\text{bac}} \) followed a single exponential giving rise to the time constants \( \tau_d \) (see Fig. 5). The values of \( \tau_d \) increased progressively as \( V_m \) was made less negative, i.e., the less negative \( V_m \), the slower was the deactivation of \( I_{\text{bac}} \). The analysis of the \( \tau_d \) data led to an exponential function \( \tau_d = f(V_m) \) (see Fig. 6). The curve-fitting procedure yielded the following parameters: \( \tau_{d,0} = 7.6 \text{ s} \) (zero \( V_m \) intercept); \( V_d = -19.7 \text{ mV} \) (decay constant). Stepping \( V_m \) from \(-50 \) to \(-5 \text{ mV} \) gave rise to a 19-fold increase in \( \tau_d \). Because of inconsistent current signals, \( \tau_d \) could not be determined at \( V_m > 5 \text{ mV} \).

The kinetics of \( I_{\text{bac}} \) activation were examined with the envelope method using trains of short test pulses. The data collected indicate that \( g_{\text{bac}} \) activated in a voltage-dependent manner (see Fig. 8). The activation was faster but less complete at negative \( V_m \) and slower but more complete at positive \( V_m \). At voltages
negative to \(-15\) mV, the data were best approximated by a single exponential, but at \(-15\) mV and more positive voltages, by the sum of two exponentials. With regard to the time course of activation, the voltage sensitivity of the time constants \(\tau_{\text{a1}}\) and \(\tau_{\text{a2}}\) was largely different (see Fig. 9A). The function \(\tau_{\text{a1}} = f(V_m)\) was shallow (●) and yielded a maximum of \(\approx 0.6\) s at around \(V_m = -20\) mV. It declined partially at more negative \(V_m\) and more completely at more positive \(V_m\). In contrast, the function \(\tau_{\text{a2}} = f(V_m)\) was steep (○) and showed a maximum of \(\approx 4.9\) s at around \(V_m = 15\) mV. It decreased sharply at more negative \(V_m\) and moderately at more positive \(V_m\). Hence, the functions \(\tau_{\text{a1}} = f(V_m)\) and \(\tau_{\text{a2}} = f(V_m)\) characterize a fast and slow process of activation, respectively. With regard to the extent of activation, the voltage sensitivity of the fast and slow process, i.e., \(C_1 = f(V_m)\) and \(C_2 = f(V_m)\), respectively, was distinctly different. At \(V_m = 40\) mV, i.e., the most positive voltage that yielded reliable data, \(C_1\) and \(C_2\) both approached a maximum, i.e., \(C_{1,\text{max}}\) and \(C_{2,\text{max}}\). Under this condition, \(C_{1,\text{max}}\) was larger than \(C_{2,\text{max}}\). The respective contributions were 58 and 42%, respectively. Moreover, the function \(C_1 = f(V_m)\) was less voltage-sensitive than the function \(C_2 = f(V_m)\) (C1 data/C2 data): \(V_{m,0} = 3.2\) \(\pm \) 9.6 mV; \(z = 2.0/6.8\) (see Eq. 5 and legend to Fig. 9). Furthermore, the values of \(C_1\) and \(C_2\) at \(V_m = 0\) mV were 0.20 and 0.34, respectively. This supports the view that a sizable fraction of hemichannels is not in the open state at \(V_m = 0\) mV (see Fig. 9B). This is consistent with the finding that at \(V_m = 0\) mV, \(g_{\text{bc,ss}}\) is substantially different from \(g_{\text{bc,ms}}\) (see Fig. 4; see also Voltage Dependence of Hemichannel Currents).

It is generally accepted that gap junction channels and hemichannels exhibit two prominent conductance states that obey the scheme 'main state ⇔ residual state' (cf. Vogel & Weingart, 1998). Moreover, it has been proposed that changes in \(g_{\text{bc,ss}}\) are due to reversible first-order processes whose forward and backward rates are unique functions of voltage (Harris et al., 1981). Therefore, the current change at a given voltage is expected to proceed with the same rate, irrespective of the voltage from which the process begins. This means that the time constants \(\tau_{\text{d}}\) and \(\tau_{\text{s}}\) ought to describe a unique function \(\tau = f(V_m)\). However, our results indicate that this is not the case. It turned out that \(I_{\text{bc,ss}}\) activation exhibits two time constants giving rise to \(\tau_{\text{a1}} = f(V_m)\) and \(\tau_{\text{a2}} = f(V_m)\). Neither of these functions represents a subset of \(\tau_{\text{a1}} = f(V_m)\) (compare Figs. 6 and 9A). For example, at \(V_m = -30\) mV, a condition where \(\tau_{\text{d}}\) and \(\tau_{\text{s}}\) are defined, \(\tau_{\text{d}} = 1.7\) s and \(\tau_{\text{s}} = 0.64\) s; or at \(V_m = -5\) mV, a condition where \(\tau_{\text{d}}, \tau_{\text{a1}}\) and \(\tau_{\text{a2}}\) are defined, \(\tau_{\text{d}} = 6.5\) s, \(\tau_{\text{a1}} = 0.39\) s and \(\tau_{\text{a2}} = 3.5\) s.

The time constants of hemichannel deactivation and activation, i.e., \(\tau_{\text{bc,cl}}\) and \(\tau_{\text{bc,ca}}\), correspond to the time constants of gap junction channel inactivation and recovery, i.e., \(\tau_{\text{cl}}\) and \(\tau_{\text{ca}}\). This convention has to be kept in mind in the following discussion. A first question to be discussed is: can the kinetics of \(I_{\text{bc,cl}}\) deactivation explain the kinetics of \(I_{\text{cl}}\) inactivation? A comparison of Cx45 hemichannel and gap junction channel data indicates that \(I_{\text{bc,cl}}\) deactivates with a single time constant at each voltage examined, i.e., \(\tau_{\text{bc,cl}}\). While \(I_{\text{cl}}\) inactivates with a single time constant at small voltages, i.e., \(\tau_{\text{cl,cl}}\), and two time constants at large voltages, i.e., \(\tau_{\text{cl,cl}}\) and \(\tau_{\text{cl,ca}}\) (cf. Barrio et al., 1997; Bader et al., 2003; but see also Moreno et al., 1995). The latter may reflect concomitant gating of both hemichannels of a gap junction channel (Baran & Weingart, 2000). However, data comparison indicates that the values of \(\tau_{\text{bc,cl}}\) are inconsistent with those of \(\tau_{\text{cl,cl}}\), despite the assumption that half of the transjunctional voltage is sensed by each hemichannel. Specifically, \(\tau_{\text{bc,cl}} < \tau_{\text{cl,cl}}\) for a transjunctional voltage \(V_j < 40\) mV and \(\tau_{\text{bc,cl}} > \tau_{\text{cl,cl}}\) for a \(V_j > 40\) mV. This discrepancy suggests that hemichannel deactivation modifies the voltage sensitivity of the hemichannels. Another explanation may be that deactivation of a hemichannel and inactivation of a gap junction channel provoke a different voltage profile. While the former senses the entire voltage drop, the latter senses the voltage drop across both hemichannels in series, i.e., one that undergoes gating and hence switches from the open to the residual state, and one that remains in the open state.

Another question to be discussed is: what is the relationship between the kinetics of \(I_{\text{bc,cl}}\) activation and the kinetics of \(I_{\text{cl}}\) recovery? An exploratory study yielded results that contradict our results. Experiments performed on injected single oocytes expressing rat Cx46 or chicken Cx56 yielded a reasonable agreement between the time course of \(I_{\text{bc,cl}}\) activation and \(I_{\text{cl}}\) recovery determined at a single voltage, i.e., \(V_m = 100\) mV (Ebihara et al. 1995). As outlined above, we failed to establish such a correlation. The reason for this discrepancy is unclear. A possibility is that the similarity of the time course of \(I_{\text{bc,cl}}\) activation and \(I_{\text{bc,cl}}\) recovery was unique to the voltage examined. Interestingly, experiments performed on pairs of hamster ventricular cells yielded a different result (Wang et al., 1992). These authors reported that \(\tau_{\text{ij}}\) was different from \(\tau_{\text{ij}}\) (\(V_j = 80\) mV: \(\tau_{\text{ij}} = 430\) ms; \(\tau_{\text{ij}} = 300\) ms), a finding that is consistent with our observations on Cx45 hemichannels.

In conclusion, deactivation and activation of Cx45 hemichannels are best described by a single exponential and the sum of two single exponentials, respectively. Moreover, the time constants of the two processes do not coincide in a voltage plot. This suggests that activation and deactivation do not follow a simple reversible reaction scheme governed by first-order voltage-dependent processes. Hence, the formalism proposed for gap junction channels in amphibian blastomeres (Harris et al., 1981) has to be
extended to include more than two channel states. Such a model may then account for the time-dependent conductance changes of hemichannels and gap junction channels consisting of Cx45 and other connexins.

Biological Significance

It is generally thought that gap junction hemichannels are silent structures that serve as precursors to establish gap junction channels. This notion assumes that they acquire their functional role only after docking with each other. However, recent findings indicate that this concept may need revision. There is evidence that Cx43 gap junction hemichannels in ventricular myocytes open up in the presence of low extracellular Ca\(^{2+}\) or during metabolic inhibition (Kondo et al., 2000). These observations complement earlier reports that elevated cytoplasmic Ca\(^{2+}\) (Rose & Loewenstein, 1976; Noma & Tsuboi, 1987) or reduced ATP\(^{2-}\) (Sugiura et al., 1990) affect the transfer properties of gap junction channels (for further references, see Harris, 2001). The relevance of these regulatory mechanisms of hemichannels may be that metabolically impaired cells take up Ca\(^{2+}\) via hemichannels and thereby enhance functional uncoupling from adjacent cells, i.e., ATP\(^{2-}\) depletion may open up hemichannels and force gap junction channels to close. This would provide a metabolic strategy to separate healthy cells from injured cells and hence save energy. Such a mechanism may be crucial during cellular insults, the outcome being dependent on the ATP\(^{2-}\) and Ca\(^{2+}\) sensitivity of the connexins.

In the context of cardiac electrophysiology, the regulatory role of Ca\(^{2+}\) and ATP\(^{2-}\) on gap junction channels and hemichannels may have the following potential impact. The opening of hemichannels brought about by reducing [Ca\(^{2+}\)], and/or [ATP\(^{2-}\)], leads to an increase in $g_{\text{leak}}$. This increase in membrane conductance short-circuits the excitatory inward current of the action potentials, thereby impairing $dV_{\text{m}}/dt_{\text{max}}$, the maximal upstroke velocity of action potential (cf. Kléber et al., 2004). This in turn leads to a decrease in conduction velocity of the electrical impulse, $0$. Likewise, the closing of gap junction channels brought about by elevating [Ca\(^{2+}\)], and/or reducing [ATP\(^{2-}\)], leads to a decrease in $g_{\text{leak}}$ and thereby decreases the functional activity. Since changes in $dV_{\text{m}}/dt_{\text{max}}$ or $g_{\text{leak}}$ act independently and synergistically on $0$, small changes have already sizable effects on $0$. The ionic and metabolic requirements that lead to such detrimental effects prevail during cardiac ischemia (cf. Kléber et al., 2004) and hence are relevant for many disease circumstances.

These considerations provoke the following generalized picture. Under physiological conditions, gap junction channels are usually open, while hemichannels are closed. Conversely, under pathophysiological condition, hemichannels open up, while gap junction channels close.

We thank D. Lüthi for expert technical assistance and H. Imboden, Department of Cell Biology, University of Bern for valuable discussions. The cells were provided by K. Willecke, Institute of Genetics, University of Bonn, Germany. This work was supported by the Swiss National Science Foundation (31-55297.98 and 31-67230.01 to R.W.).

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4.2. Pitfalls when examining gap junction hemichannels: interference from volume regulated anion channels
Pitfalls when examining gap junction hemichannels: interference from volume-regulated anion channels

Received: 21 December 2005 / Accepted: 5 January 2006
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Abstract Human HeLa cells transfected with mouse connexin45 were used to explore the experimental conditions suitable to measure currents carried by gap junction hemichannels. Experiments were performed with a voltage-clamp technique and whole-cell recording. Lowering $[Ca^{2+}]_o$ from 2 mM to 20 nM evoked an extra current, $I_m$, putatively carried by Cx45 hemichannels. However, the variability of $I_m$ (size, voltage sensitivity, kinetics) suggested the involvement of other channels. The finding that growth medium in the incubator increased the osmolarity with time implied that volume-regulated anion channels (VRAC) may participate. This assumption was reinforced by the following observations. On the one hand, keeping $[Ca^{2+}]_o$ normal while the osmolarity of the extracellular solution was reduced from 310 to 290 mOsM yielded a current characteristic of VRAC; $I_{VRAC}$ activated/deactivated at negative/positive voltage, giving rise to the conductance functions $g_{VRAC,in} = f(V_m)$ (instaneous; $V_m$, membrane potential) and $g_{VRAC,os} = f(V_m)$ (ss; steady state). Moreover, it was reversibly inhibited by mibebradil, a CF channel blocker (binding constant $K_B = 38 \mu M$, Hill coefficient $n = 12$), but not by the gap junction channel blocker 18α-glycyrrhetinic acid. On the other hand, minimizing the osmotic imbalance while $[Ca^{2+}]_o$ was reduced led to a current typical for Cx45 hemichannels; $I_{os}$ activated/deactivated at positive/negative voltage. Furthermore, it was reversibly inhibited by 18α-glycyrrhetinic acid or palmitoleic acid, but not by mibebradil. Computations based on $g_{VRAC,in} = f(V_m)$ and $g_{os} = f(V_m)$ indicated that the concomitant operation of both currents results in a bell-shaped conductance–voltage relationship. The functional implications of the data presented are discussed. Conceivably, VRAC and hemichannels are involved in a common signaling pathway.

Keywords Gap junction hemichannel · Connexin45 · Volume-regulated anion channel · Electrophysiology · Pharmacology

Introduction

Gap junctions are aggregates of channels offering a direct pathway for the exchange of ions and small molecules between adjoining cells. Each channel consists of two hemichannels in series, and each hemichannel (connexon) is made of six subunits (connexins) forming an aqueous pore. Gap junctions are dynamic structures [23]. Formation of their channels involves docking of preformed hemichannels located in the cell membrane of adjacent cells; breakdown of channels occurs via endocytosis and degradation.

Cell pairs are suitable for studying gap junction channels with a dual voltage-clamp technique (see [13]). It enables one to determine the conductive and kinetic channel properties. Single cells are convenient for examining hemichannels with a single voltage-clamp method [28]. It allows one to assess the respective properties of hemichannels and gain insight into functional aspects of gap junction channels not accessible to cell-pair studies. Solitary hemichannels embedded in cell membranes are usually closed under physiological conditions. To study their properties, they have to be opened using specific experimental protocols, i.e., lowering $[Ca^{2+}]_o$ or altering the membrane potential (see [13]). When examining the electrical properties of hemichannels, a potential problem is the interference from other channels. Various preparations have been used in the past to examine hemichannels, e.g., isolated primary cells [7, 14], injected oocytes [10, 26] or transfected cell lines [2, 16, 27, 28]. In some cases, the measures taken to limit the contribution of other channels may have been scant. Recent evidence suggests that hemichannels residing in cell membranes can be opened under physiological or pathophysiological conditions [14, 15, 24]. In this study, we have optimized the conditions to determine the electrical properties of hemichannels using
cells with a high input resistance and solutions with channel blockers to minimize interference from other currents. For this purpose, we have used HeLa cells transfected with mouse connexin45. They possess a limited selection of intrinsic channels, i.e., potassium channels [8, 21, 22] and a chloride channel [9, 19, 22]. It turned out that small osmotic disturbances are sufficient to activate an extra current. Electrophysiological and pharmacological experiments indicated that this current is carried by volume-regulated anion channels (VRAC). Since its properties are mirror images of those of hemichannel currents, caution is required when examining the latter. A preliminary report has been published before [1].

Methods

Cells and culture conditions

Human HeLa cells transfected with cDNA coding for mouse connexin45 [11] and wild-type HeLa cells were grown in DMEM containing 10% FCS, 100 µg/ml streptomycin and 100 U/ml penicillin. Transfectants were selected with 0.5–1 µM puromycin (Sigma). For experiments, the cells were harvested in DMEM containing 10% FCS (2.8 x 10^8 to 10^9 cells/ml) and seeded onto sterile glass coverslips placed in culture dishes (5 x 10^4 cells/cm²). Electrical measurements were performed 1–2 days later.

Solutions

Experiments were carried out in KCl-rich solution containing normal Ca²⁺ [in millimolar: KCl, 140; NaCl, 4; CaCl₂, 2; MgCl₂, 1; HEPES, 5 (pH 7.4); glucose, 5; pyruvate, 2; CsCl₂, 2; BaCl₂, 1; TEA–Cl⁻, 2] or low Ca²⁺ [in millimolar: KCl, 140; NaCl, 4; CaCl₂, 1; MgCl₂, 1; MgATP, 3; HEPES, 5 (pH 7.2); EGTA, 5 (free Ca²⁺ ~45 nM or pCa=7.4); CsCl₂, 2; BaCl₂, 1; TEA–Cl⁻, 2]. Ba²⁺, Ca²⁺ and TEA– were added to block K⁺ currents. To prepare stock solutions, 18α-glycyrrhetinic acid (G-8503; Sigma) and palmotolic acid (P-9417; Sigma) were dissolved in DM SO (20 mM) and hexane (20 mM), respectively; mibebradil (gift from La Roche, Basel, Switzerland) was dissolved in H₂O (20 mM). Initially, the osmolarity of the salt solutions was not adjusted (see “Initial observations” in “Results”). Later on, this was done by adding mannitol. The osmolarity of growth medium was 310 mOsm and served as standard. Osmolarity was measured with an osmometer (3MO; Advanced Instruments, Needham Heights, MA, USA).

Electrical measurements

Coverslips with adherent cells were transferred to an experimental chamber mounted on an inverted microscope (Diaphot-TMD; Nikon, Nippon Kogaku, Tokyo, Japan). The chamber was superfused with Ca²⁺-containing solution at room temperature (22–25°C). Patch pipettes were pulled from glass capillaries (GC150F-10; Harvard Apparatus, Edenbridge, UK) with a horizontal puller (DMZ-Universal; Zeitz Instruments, Augsburg, Germany). To reduce capacitance, the pipette tips were coated with silicon elastomer (Sylgard 184; Dow Corning, Wiesbaden, Germany). When filled with solution, the pipettes had DC resistances of 3–5 MΩ. Experiments were carried out on single cells using a voltage-clamp method and whole-cell recording [28]. Patch pipettes connected to an amplifier (EPC 7; HEKA Elektronik, Lambrecht/Pfalz, Germany) were manoeuvred with a micromanipulator (MP-258; Sutter Instrument, Novato, CA, USA).

Signal recording and analysis

The signals were filtered at 1 kHz (8-pole Bessel) and digitized at 3 kHz (ITC-16; Instrutech, Port Washington, NY, USA). Data acquisition and analysis were done with PULSE/PULSFIT (HEKA Elektronik), curve fitting and statistical analysis with SigmaPlot and SigmaStat (Jandel Scientific, Erkrath, Germany), respectively.

Results

Initial observations

After establishing the whole-cell recording condition in solution containing normal Ca²⁺, the bath was superfused with low-Ca²⁺ solution. In transfected HeLa cells, this was expected to elicit a current carried by Cx45 hemichannels [2]. The extra current observed varied considerably. In some cells, it was significant, in others marginal. This may reflect a change in Cx45 expression during the cell cycle [3]. However, in some cells, the extra current showed a time-dependent decay at negative Vm, in others at positive Vm (Fig. 1). Starting from a holding potential Vh=0 mV, Vm was first depolarized (prepulse) and then hyperpolarized (test pulse) or vice versa. Figure 1a shows a cell whose Im increased slightly with time at positive voltage (Vm=30 mV) and decayed significantly with time at negative voltage (Vm=-40 mV). Conversely, Fig. 1b shows a cell whose Im increased marginally at negative voltage (Vm=-90 mV) and declined prominently at positive voltage (Vm=90 mV). In both cases, prior to the intervention, Im was small and time-independent (superimposed traces). The diverse behaviour of Im may indicate the presence of two types of connexins. However, this possibility can be ruled out. Immunohistochemistry has revealed that wild-type HeLa cells express the same connexin as the transfected cells examined, i.e. Cx45, but at negligible level [11].

In some transfecteds, currents similar to those in Fig. 1b already developed in solution with normal Ca²⁺. Yet, it is generally thought that hemichannels are closed under this condition. Moreover, some wild-type HeLa cells showed such currents in low-Ca²⁺ solution. This would be con-
sistent with the report that pairs of these cells exhibit intercellular currents attributable to intrinsic Cx45 [30]. However, their low incidence renders it unlikely.

To solve the puzzle, an involvement of other channels was considered. HeLa cells have two types of inwardly rectifying K⁺ channels of low unitary conductance [8, 21, 22]. However, interference from these channels could be excluded because the solutions contained blocking agents such as Cs⁺, TEA⁺ and Ba²⁺ (see [28]). Searching for other channels, we have measured the osmolarity of the solutions. A key finding was that fresh and used growth medium showed a different osmolarity. It increased by ~6% in 48 h in the incubator, despite the water-saturated atmosphere. Hence, switching from growth medium to salt solutions imposed an osmotic imbalance, suggesting the interference from VRAC ([9, 19]; see also [18]).

Volume-regulated anion channels

To explore the involvement of VRAC, the medium in the culture dishes was replaced by fresh medium 2–3 h prior to an experiment. This enabled the cells to readjust to the reference osmolarity, thus avoiding osmotic effects when transferring cells to the experimental chamber. Transfected HeLa cells pretreated in this way failed to develop an extra current in solution with normal Ca²⁺. However, when superfused with low-Ca²⁺ solution, an extra current developed persistently comparable to that in Fig. 1a.

Next, the focus was on salt solutions. The salines with normal and low Ca²⁺ as well as the pipette solution were adjusted to the reference osmolarity to minimize osmotic shifts when changing solution. The growth medium in the culture dishes was again replaced prior to the use of cells. After a control period of 1 min, the solution with normal Ca²⁺ and osmolarity was replaced by solution with normal Ca²⁺ but reduced osmolarity (290 mOsm). During control, hyperpolarization and depolarization led to a small constant inward and outward current, respectively. During the intervention, an extra current evolved with an increasing inward and a decreasing outward component, respectively, similar to that in Fig. 1b. As illustrated in Fig. 2, the development of the extra current, plotting as a function of time the amplitude of the maximal inward current, \( I_{VRAC_{in}}, \) the instantaneous outward current, \( I_{VRAC_{inst}}, \) and the steady-state outward current, \( I_{VRAC_{ss}}, \) suggests the interference from VRAC. Because it vanished after osmotic adjustment of the growth medium and the salt solutions, it cannot be serum-mediated.

Pharmacology of \( I_{VRAC} \)

Pharmacological experiments were done to further qualify the current induced by osmotic changes. Mibefradil, a VRAC blocker, seemed appropriate for this purpose [18, 20]. Figure 3 illustrates an experiment. The bipolar pulse protocol (see Fig. 1b) was applied every 30 s. At time \( t=0 \) min, the osmolarity of the solution was reduced from 310 to 290 mOsm. Shortly after the intervention, the three current components begun to grow; \( I_{VRAC_{max}} \) and \( I_{VRAC_{inst}} \) increased significantly, \( I_{VRAC_{ss}} \) marginally. Exposure to 40 μM mibefradil at time \( t=5 \) min reduced \( I_{VRAC_{max}} \) and \( I_{VRAC_{inst}} \). Upon washout of the blocker at \( t=10 \) min, the current components increased again. During the intervention, \( I_{max} \) underwent a small maintained increase which may reflect a minor leak. The incomplete recovery of \( I_{VRAC_{max}} \) and \( I_{VRAC_{inst}} \) during washout of mibefradil and their increase beyond the level prior to the intervention are consistent with this explanation. Four additional experiments yielded similar results. They suggest that the extra current was carried by VRAC.

Fig. 1 Recruitment of extra currents. a Upper panel: pulse protocol. Starting from 0 mV, \( V_m \) was depolarized to 30 mV for 10 s (prepulse), then hyperpolarized to -40 mV for 2 s (test pulse). Lower panel: currents \( I_m \) in control solution (small signal) and after 5 min in low-Ca²⁺ solution (large signal). b Upper panel: pulse protocol, \( V_m \) was hyperpolarized to -90 mV for 5 s, then depolarized to 90 mV for 5 s. Lower panel: currents \( I_m \) in control solution (small signal) and after ~5 min in low-Ca²⁺ solution (large signal).
plotted vs log [mibebradil] (Fig. 4). The smooth curve corresponds to the best fit of data to the equation

\[
g_{VRAC} = \frac{1}{1 + (K_d/\text{[mibebradil]})^n}
\]

for the following parameters: \(K_d=38 \mu M\) (binding constant), \(n=12\) (Hill coefficient). Complementary experiments revealed that \(I_{VRAC}\) is not affected by 40 \(\mu M\) 18\(\alpha\)-glycerylhetinic acid and barely reduced by 50 \(\mu M\) palmitoleic acid (12±2.7%, \(n=8\)), doses fully blocking gap junction hemichannels (see “Pharmacology of \(I_{in}\”)).

Voltage sensitivity of \(I_{VRAC}\)

To study the voltage dependence of \(I_{VRAC}\), cells were subjected to the same osmotic intervention (see above). Starting from \(V_m=0\) mV, \(V_m\) was hyperpolarized to \(-90\) mV for 3 s and then depolarized to different levels for 5 s at increments of 10 mV (Fig. 5a). The prepulse served to evoke a maximal \(I_{VRAC}\), the test pulse to study the voltage dependence. Figure 5b shows a family of records. The induced current showed a distinct deactivation at positive \(V_m\). For analysis, the amplitudes of \(I_{VRAC_{\text{inst}}}\) and \(I_{VRAC_{\text{ss}}}\) were determined at the beginning and end of each test pulse. Figure 5c illustrates the current–voltage relationships. Over the \(V_m\) range examined, \(I_{VRAC_{\text{inst}}}\) (○) showed a slight outward rectification with a curved segment at negative voltage and a nearly linear segment at positive voltage (conductance 18.6 nS). In contrast, \(I_{VRAC_{\text{ss}}}\) (●) showed a prominent decline at voltages above 30 mV.

Figure 6a illustrates the normalized functions \(g_{VRAC_{\text{inst}}} = f(V_m)\) (○) and \(g_{VRAC_{\text{ss}}} = f(V_m)\) (●) summarizing the data from four cells. To normalize the \(g_{VRAC_{\text{inst}}}\) data, the conductance extrapolated to \(V_m=0\) mV was used as reference. It was obtained by averaging the values of \(g_{VRAC_{\text{inst}}}\) at \(V_m=±\)

---

**Fig. 2** Effects of osmolarity on recruitment of extra currents putatively carried by VRAC. A bipolar voltage pulse (prepulse: −90 mV, 5 s; test pulse: 90 mV, 5 s) was applied every 30 s. At time \(t=0\) min, solution with normal Ca\(^{2+}\) (2 mM) and of normal osmolarity (310 mOsm) was replaced by solution of reduced osmolarity (290 mOsm). ▲, \(I_{\text{VRAC_{max}}}\) (maximal inward current associated with prepulse); ○, \(I_{\text{VRAC_{inst}}}\) (instantaneous outward current at beginning of test pulse); ●, \(I_{\text{VRAC_{ss}}}\) (steady-state outward current at end of test pulse).

**Fig. 3** Pharmacological identification of currents putatively carried by VRAC. Time-course study of current components. ▲, \(I_{\text{VRAC_{max}}}\) (maximal inward current associated with prepulse); ○, \(I_{\text{VRAC_{inst}}}\) (instantaneous outward current at beginning of test pulse); ●, \(I_{\text{VRAC_{ss}}}\) (steady-state outward current at end of test pulse). Interventions: time \(t=0\) min, reduction in osmolarity of bath solution from 310 to 290 mOsm; \(t=5\) min, addition of 40 \(\mu M\) mibebradil; \(t=10\) min, washout of mibebradil.

**Fig. 4** Dose–response relation for \(I_{VRAC}\) vs mibebradil concentration. Normalized changes in \(I_{VRAC}\) were averaged and plotted against [mibebradil] on log scale. Symbols correspond to mean values±1 SEM (\(n=11\)). Continuous curve: least-squares fit of data to Hill equation: \(K_d=38 \mu M\) (binding constant), \(n=12\) (Hill coefficient).
The analysis yielded the following parameters: $V_{RAC, \text{max}} = 39.9$ mV ($V_m$ at which $g_{VRAC ss}$ is half-maximally deactivated), $g_{VRAC, \text{max}} = 1.01$ (maximal value of $g_{VRAC ss}$), $g_{VRAC, \text{min}} = 0.18$ (minimal value of $g_{VRAC ss}$), $A = 0.32$ (constant expressing gating charge), $z = 8.1$ (unitary positive charge $q$ moving through the electric field applied; $A = q(kT)^{-1}$; $k$, Boltzmann constant; $T$, temperature in Kelvin).

VRAC currents were also studied in the presence of pipette solution containing K+ aspartate instead of KCl. This resulted in a shift of the reversal potential from $0.5 \pm 0.9$ to $22.5 \pm 3.7$ mV ($n=5$), indicating that the channels are less permeable to aspartate than Cl⁻.

**Kinetics of $I_{VRAC}$**

The current records in Fig. 5b were also used to analyse the time-course of $I_{VRAC}$ deactivation. A least-square curve-fitting indicated that $I_{VRAC}$ decreases exponentially with

![Graph showing voltage dependence of currents carried by VRAC.](image)

![Graph showing conductance and kinetic properties of VRAC.](image)
time (not shown). Figure 6b shows the analysis plotting the time constants of deactivation, \( \tau_d \), as function of \( V_m \). Over the voltage range examined, \( \tau_d \) decreased steeply as \( V_m \) was made more positive. The smooth curve corresponds to the best fit of data to an exponential,

\[
\tau_d = \tau_{d,0} \cdot e^{-\frac{V_m}{\tau_c}},
\]

(3)

using the following parameters: \( \tau_{d,0} = 317 \) s (zero \( V_m \) intercept), \( \tau_c = 11.6 \) mV (decay constant).

Gap junction hemichannels

Currents putatively carried by Cx45 hemichannels, \( I_{he} \), were elicited in solution with low Ca\(^{2+} \). The following precautions were taken to minimize the interference from \( I_{VRAC} \): (1) adjustment of the osmolarity of each solution to 310 mOsm and (2) addition of 60 \( \mu \)M mibefradil to the bath solution. The currents were studied with a bipolar pulse protocol. Starting from \( V_m = 0 \) mV, \( V_m \) was depolarized to 30 mV for 10 s and then hyperpolarized to -40 mV for 5 s. This yielded current signals similar to that in Fig. 1a. Figure 7 illustrates the recruitment of the extra current plotting the amplitude of the maximal outward current \( I_{he,max} \) (c), the instantaneous inward current \( I_{he,inst} \) (o) and the steady-state inward current \( I_{he,ss} \) (e). The extra current was absent in wild-type HeLa cells, suggesting that it is carried by Cx45 hemichannels [2].

Pharmacology of \( I_{he} \)

\( I_{he} \) was further qualified by exposure to palmitoleic acid, a gap junction channel blocker [4]. After a control period, i.e. at time \( t = 0 \) min, the extracellular Ca\(^{2+} \) concentration was reduced to elicit an \( I_{he} \). Figure 8 illustrates an experiment. The amplitude of \( I_{he,max} \) (c), \( I_{he,inst} \) (o) and \( I_{he,ss} \) (e) increased gradually. At time \( t = 7 \) min, 50 \( \mu \)M palmitoleic acid was applied. This led to a complete inhibition of the extra current. At time \( t = 11 \) min, the blocking agent was washed out. To speed up the recovery, 1 mg/ml albumin was added to the washout solution to trap the fatty acid. The amplitude \( I_{he,max} \) and \( I_{he,ss} \) reached during washout was significantly larger than prior to the drug application, suggesting that additional hemichannels were recruited during the intervention. At time \( t = 17.5 \) min, exposure to palmitoleic acid was repeated. Within 3 min, the current components were completely blocked again. Similar observations were made in seven other cells. Exposure to 40 \( \mu \)M 18α-glycyrrhetinic acid, another blocker of gap junction channels (see [13]), yielded similar results (three cells). Conversely, exposure to 60 \( \mu \)M mibefradil, a dose fully blocking \( I_{VRAC} \) (see “Pharmacology of \( I_{VRAC} \)”), had no effect on \( I_{he} \) (data not shown).

Voltage sensitivity and kinetics of \( I_{he} \)

The bipolar pulses were also used to study the electrical properties of \( I_{he} \). Starting from \( V_m = 0 \) mV, \( V_m \) was depolarized to 30 mV for 10 s and then hyperpolarized to different levels at increments of 5 to 20 mV (Fig. 9a). The duration of the test pulse varied from 7 to 40 s, enabling \( I_{he} \) to reach a steady state. Figure 9b shows superimposed

![Fig. 7 Effects of low extracellular Ca\(^{2+}\) on recruitment of currents carried by Cx45 hemichannels. A bipolar voltage pulse (prepulse: 30 mV, 10 s; test pulse: -40 mV, 5 s) was applied every 30 s to elicit the extra current, \( I_{he} \). At time \( t = 0 \) min, solution of normal Ca\(^{2+} \) (2 mM) and osmolarity (310 mOsm) was replaced by solution with low Ca\(^{2+} \) (~20 nM). The following current components were plotted against time: α, \( I_{he,max} \) (maximal outward current during prepulse); β, \( I_{he,inst} \) (instantaneous inward current at beginning of test pulse); γ, \( I_{he,ss} \) (steady-state inward current at end of test pulse).](image1)

![Fig. 8 Pharmacological identification of currents carried by Cx45 hemichannels. Time-course study of current components: α, \( I_{he,max} \) (maximal outward current during prepulse to 30 mV); β, \( I_{he,inst} \) (instantaneous inward current at beginning of test pulse to -40 mV); γ, \( I_{he,ss} \) (steady-state inward current at end of test pulse). Interventions: time \( t = 0 \) min, Ca\(^{2+} \) in the bath was reduced from 2 mM to ~20 nM; \( t = 7 \) min, 50 \( \mu \)M palmitoleic acid was washed in; \( t = 11 \) min, palmitoleic acid was washed out in presence of albumin (1 mg/ml) to speed up recovery.](image2)
traces indicating that $I_{hc}$ deactivated at negative $V_m$. Figure 9e shows the current/voltage relationships deduced from the records in Fig. 9b and others from the same experiment, plotting $I_{h_{hc,inst}}$ (○) and $I_{h_{hc,ss}}$ (●), respectively, as a function of $V_m$. The $I_{hc,inst}$ data yielded a quasi-linear relationship with deviations towards the x-axis at large $V_m$. In contrast, the $I_{hc,ss}$ data showed a pronounced decrease at negative voltages.

Current records like those in Fig. 9b were then used to establish the voltage dependence of $g_{h_{hc,inst}}$ and $g_{h_{hc,ss}}$. The result of this analysis has already been communicated [2]. Since it is essential for a comparison with the properties of $I_{V_RAC}$, the procedure is outlined briefly and the data are presented in condensed form. The values of $I_{h_{hc,inst}}$ and $I_{h_{hc,ss}}$ were used to calculate $g_{h_{hc,inst}}$ and $g_{h_{hc,ss}}$, respectively. In the case of $g_{h_{hc,inst}}$, a value extrapolated to $V_m=0$ mV served as a reference. In the case of $g_{h_{hc,ss}}$, the data were expressed as a fraction of $g_{h_{hc,inst}}$ of the same pulse. The normalized data were averaged and plotted as a function of $V_m$. Figure 10a depicts the functions $g_{h_{hc,inst}}/g(V_m)$ (dashed line) and $g_{h_{hc,ss}}/g(V_m)$ (continuous curve) derived from curve fitting, omitting the data points for clarity (replotted from [2]). The function $g_{h_{hc,inst}}(V_m)$ was linear and exhibited a small negative slope. The function $g_{h_{hc,ss}}(V_m)$ was sigmoidal. It showed a maximum close to positive and a minimum at negative $V_m$, leading to a steep transition with a positive slope (Boltzmann parameters: $V_{hc,0}=-1.08$ mV, $g_{hc,max}=1.04$, $g_{hc,min}=0.08$, $z=4.0$ [2]).

The kinetics of $I_{hc}$ have also been examined before [2]. Again, for comparison with the $I_{V_RAC}$ data, the analysis procedure and the deactivation data are summarized briefly. Figure 9b shows that $I_{hc}$ decayed exponentially with time. Curve-fitting yielded the time constants of deactivation, $\tau_d$. Figure 10b shows a plot of the function $\tau_d=f(V_m)$, omitting the data points (replotted from [2]). Over the voltage range

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**Fig. 9** Voltage dependence of $I_{hc}$. a Bipolar pulse protocol. A depolarizing prepulse from 0 to 30 mV for 10 s was followed by a variable test pulse to negative (-50, -30, and -10 mV) and positive voltage (15 and 40 mV) for 7 to 20 s. b Family of associated current traces, $I_{hc}$. The prepulse resulted in an outward current. Test pulses to negative $V_m$ gave rise to inward currents decaying with time; test pulses to positive $V_m$ led to outward currents of constant amplitude. c Plot of instantaneous current, $I_{h_{hc,inst}}$ (○), and steady-state current, $I_{h_{hc,ss}}$ (●), against $V_m$.

**Fig. 10** Conductance and kinetic properties of Cx45 hemichannels. a Plot of instantaneous conductance, $g_{h_{hc,inst}}$ (dashed line), and steady-state conductance, $g_{h_{hc,ss}}$ (continuous curve), against $V_m$. Continuous curve: least-squares fit of data to Eq. 2: $g_{h_{hc,inst}}=1.08 mV$, $g_{hc,max}=1.04$, $g_{hc,min}=0.08$, $z=4.0$. b Plot of time constant of $I_{hc}$ deactivation, $\tau_d$ against $V_m$. Continuous curve: least-squares fit of data to Eq. 3: $\tau_d=7.6 s$ (zero $V_m$ intercept), $V_f = 19.7 mV$ (decay constant). Redrawn from [2]. Data points omitted for clarity.
from −50 to 5 mV, \( \tau_d \) increased monotonically as \( V_m \) was made less negative. The curve corresponds to the best fit of data to Eq. 3: \( \tau_{d,0} = 7.6 \text{ s}, V_c = 19.7 \text{ mV} \) [2].

Concomitant operation of VRAC and Cx45 hemichannels

When studying \( I_{VRAC} \) in appropriate solution (normal Ca\(^{2+}\), reduced osmolarity), \( g_{VRAC,max} \) determined at \( V_m = -50 \text{ mV} \) was 16.2±0.6 nS (\( n = 4 \)). When examining \( I_{hc} \) in appropriate solution (low Ca\(^{2+}\), normal osmolarity), \( g_{hc,max} \) determined at \( V_m = 30 \text{ mV} \) was 13.3±2.7 nS (\( n = 8 \)). If one considers the unitary conductance of the respective channels at these voltages, i.e. ~10 and ~53 pS (T. Voets, unpublished, quoted in [18, 27]), this corresponds to 1.600 and 250 operational VRAC and Cx45 hemichannels, respectively.

On the basis of these data and those on \( V_m \) dependence of \( g_{VRAC,ss} \) and \( g_{hc,ss} \) (see Figs. 6a and 10a), an attempt was made to simulate the concomitant presence of \( I_{VRAC} \) and \( I_{hc} \). This situation prevailed when \( I_{hc} \) was studied with osmotically unbalanced solutions (see “Initial observations”). Figure 11 illustrates the simulation data plotting the absolute values of \( g_{m,ss} \) against \( V_m \). The continuous curves a and e represent the functions \( g_{VRAC,ss} = f(V_m) \) and \( g_{hc,ss} = f(V_m) \), respectively, calculated from the experimental data (mean values of \( g_{VRAC,max} \) and \( g_{hc,max} \) given above, and Boltzmann parameters from Figs. 6a and 10a). These functions were then used to compute the behaviour of \( g_{m,ss} \) in the case of a simultaneous operation of VRAC and Cx45 hemichannels. The interrupted curves illustrate the conductance functions assuming the following ratios of \( g_{VRAC,max}/g_{hc,max} \): 75/25% (b), 50/50% (c); 25/75% (d). They were calculated from the equation

\[
\begin{align*}
g_{m,ss} = f(V_m) \\
= p[g_{m,ss} = f(V_m)]_{VRAC} + q[g_{m,ss} = f(V_m)]_{hc}
\end{align*}
\]

where \( p \) and \( q \) are the relative weights of \( g_{VRAC,max} \) and \( g_{hc,max} \), respectively [6]. Instead of sigmoidal, these functions are bell-shaped and exhibit a maximum at \( V_m \approx 20 \text{ mV} \). The filled circles connected by solid lines represent the data from an early experiment examining Cx45 hemichannels (see “Initial observations”). The contour of this plot was also bell-shaped. Hence, it resembles those calculated for a simultaneous operation of VRAC and Cx45 hemichannels.

**Discussion**

Under certain experimental conditions, Cx45-HeLa cells exhibit two complementary currents. One of them is carried by anions (primarily Cl\(^-\) in the present context) and deactivates at positive \( V_m \). It is attributable to VRAC (see [18]). The other one is carried by anions and cations (primarily K\(^+\) and Cl\(^-\)) and deactivates at negative \( V_m \). It involves Cx45 hemichannels [2, 27]. Concomitant operation of both channels provokes complex currents and may lead to erroneous interpretations.

**Volume-regulated anion channels**

**Biophysical characterization** To examine currents carried by VRAC, cells were subjected to a mild osmotic intervention, i.e. lowering the osmolarity of the bath solution from 310 to 290 mOsm. This evoked constant inward currents at negative \( V_m \) and outward currents with a prominent deactivation at positive \( V_m \) (see Fig. 5b). The function \( g_{VRAC,ini} = f(V_m) \) was slightly sigmoidal and showed a positive slope, while \( g_{VRAC,dec} = f(V_m) \) was clearly sigmoidal and yielded a negative slope \( (V_m)_{0} = -39.9 \text{ mV}, \ z = 8.1 \) (see Fig. 6a). The maximal \( g_{VRAC,ini} \) at negative \( V_m \) reflects the total number of channels in the open state, and the minimal \( g_{VRAC,dec} \) at positive \( V_m \) an incomplete deactivation of VRAC and/or a contribution of background currents. The kinetic analyses revealed that \( I_{VRAC} \) deactivates exponentially with time at positive \( V_m \), giving rise to single time constants, \( \tau_d \) (see Fig. 5b). The more positive the \( V_m \), the faster was the deactivation. Over the voltage range yielding reliable data, i.e. 40 to 80 mV, the function \( g_{VRAC,dec} = f(V_m) \) was exponential (zero \( V_m \) intercept \( \tau_{d,0} = 317 \text{ s}, \text{ decay constant } V_c = 11.4 \text{ mV} \) (see Fig. 6b).
conclusion, since VRAC are open at physiological $V_m$ and deactivate at $V_m$≥25 mV, they are expected to affect the properties of non-excitabile and excitabile membranes, i.e. the resting potential and/or the action potential, despite the small unitary conductance.

Similar $I/V$ relations of $I_{VRAC,inst}$ have been reported for different types of cells ([9, 19]; B. Nilius and J. Prenen, unpublished observation, quoted in [18]). However, so far no kinetic data have been made available. Recently, it has been reported that single VRAC exhibit an $I/V$ relationship with outward rectification (T. Voets, unpublished, see [18]). This offers an explanation for the non-linear relationship $I_{VRAC,inst}=f(V_m)$. Interestingly, it has been observed that $I_{VRAC}$ decreases in size when cells switch from proliferation to differentiation (see [18]). Moreover, it was found that Cl$^-$ currents alter during the cell cycle, being high in G1-phase and low in S-phase and increasing again in M-phase [5, 31]. This may explain the variability of $I_{VRAC}$ in our study carried out on cells of non-synchronized cultures.

Replacing Cl$^-$ in the pipette solution against aspartate, we found a shift of the reversal potential of $I_{VRAC}$ towards positive voltages. This suggests that these channels are more permeable to Cl$^-$ than aspartate (see “Voltage sensitivity of $I_{VRAC}$”). This preference is characteristic for VRAC channels (see [18]).

Ionic characterization An odd current appeared occasionally when Cx45-HeLa cells were exposed to solutions aimed at examining $I_{he}$ (see “Initial observations”). It turned out that this was caused by osmotic perturbations. When cells were grown in the incubator, the osmolarity of the medium increased gradually (~3% per day). Subsequent exposure to salt solutions introduced an osmotic stress which caused cell swelling via water influx and activation of VRAC (see [18]). Such small osmotic shifts were sufficient to evoke $I_{VRAC}$.

Pharmacological characterization Mibefradil is a useful tool to block VRAC [18, 20]. We found that it inhibited $I_{VRAC}$ in a dose-dependent and reversible manner (see Fig. 4). It had no effect on Cx45 hemichannels. Hence, 60 μM mibefradil was routinely added to the bath solution when $I_{he}$ was examined (see “Cx45 hemichannels”). Conversely, $I_{VRAC}$ was not affected by 40 μM 18α-glycyrrhetinic acid and only weakly impaired by 50 μM palmitoleic acid, doses which fully block gap junction channels and hemichannels (see [13]).

Cx45 hemichannels

Biophysical characterization To study currents carried by Cx45 hemichannels, $I_{he}$, cells were exposed to low-Ca$^{2+}$ solution. This evoked a current whose voltage sensitivity was a mirror image of $I_{VRAC}$ (see Fig. 9). Depolarization gave rise to outward currents which activated slowly with time; hyperpolarization yielded inward currents with a prominent deactivation. The function $g_{he,inst}=f(V_m)$ was linear and exhibited a negative slope, while $g_{he,ss}=f(V_m)$ was sigmoidal and grew with positive $V_m$ (Boltzmann parameters: $V_m^{0}=-1.08$ mV, $z=-4.0$; see Fig. 10a). The maximal $g_{he,ss}$ at positive $V_m$ corresponds to the channels in the open state, and the minimal $g_{he,ss}$ at negative $V_m$ to incomplete channel closure and/or incomplete deactivation of $I_{he}$ [2]. However, a contribution of background currents cannot be excluded. A recent study on Cx45 hemichannels reported a similar relationship $g_{he,ss}=f(V_m)$ [27]. Comparison of $I_{he,ss}$ and $I_{VRAC,ss}$ data indicates that gating of Cx45 hemichannels is less voltage-sensitive than gating of VRAC. The kinetic studies revealed that $I_{he}$ deactivates exponentially with time, giving rise to single time constants, $\tau_d$. The more negative the $V_m$ the more rapid was the deactivation. Over the range of $V_m$ which yielded reliable data, i.e. -40 to -5 mV, the function $\tau_d=f(V_m)$ was exponential ($\tau_d^{0}=7.6$ s, $V_m=19.7$ mV; see Fig. 10b). Hence, deactivation of $I_{he}$ is less voltage-sensitive than deactivation of $I_{VRAC}$. In conclusion, since most Cx45 hemichannels are in the residual state at physiological $V_m$ [27], they are likely to have a small effect on the properties of non-excitabile and excitabile membranes. Nonetheless, few open channels of a sizable unitary conductance may already affect $V_m$.

It has been reported that the unitary currents of Cx45 hemichannels are voltage-sensitive [27]. This suggests that the negative slope of the function $g_{he,inst}=f(V_m)$ is an intrinsic property of Cx45 hemichannels.

Ionic characterization To activate a maximal number of Cx45 hemichannels, [Ca$^{2+}$]o had to be reduced from 2 mM to ~20 nM (P. Bader, R. Weingart and M. Egger, in preparation). This finding differs from a recent study on Cx45 hemichannels performed in solution nominally free of Ca$^{2+}$ [27]. Without precautions, such solutions have a free [Ca$^{2+}$]≥10 μM [17], a value much larger than [Ca$^{2+}$]o used in our experiments. This difference may contribute to the larger $g_{he,max}$ found in the present study (13.3 vs 5.9 NS). If we assume that Cx45 gap junction channels exhibit a Ca$^{2+}$ sensitivity similar to that of other gap junction channels (see [13]), this suggests that Cx45 hemichannels are more sensitive to Ca$^{2+}$ than Cx45 gap junction channels. Alternatively, Cx45 gap junction channels and hemichannels may be more sensitive to Ca$^{2+}$ than other channels and hemichannels.

A recent paper reported that osmotic stress, like metabolic inhibition, elicited a current carried by Cx43 hemichannels [14]. In light of our data, this raises the question whether the current evoked was carried by VRAC rather than hemichannels.

Pharmacological characterization Palmitoleic acid blocks gap junction channels [4]. It inhibited Cx45 hemichannels
in a dose-dependent manner; complete block of $I_{he}$ was obtained at 50 μM (see Fig. 8). $I_{he}$ was also inhibited by 40 μM 18α-glycyretinic acid which acts on gap junction channels and hemichannels [13]. In contrast, Cx45 hemichannels were not affected by mibebradil up to 60 μM (see “Pharmacology of $I_{he}$” in “Results”). Recently, it has been reported that some Cl channel blockers, i.e. flufenamic acid and NPPB, also inhibit Cx50 and Cx46 hemichannels [12]. Since the latter agent also blocks VRAC (see [18]), caution is suggested when mibebradil is used for hemichannel studies.

Concurrent operation of VRAC and Cx45 hemichannels

VRAC and Cx45 hemichannels have similar and dissimilar properties. VRAC are outward rectifying and deactivate at positive $V_m$, Cx45 hemichannels are inward rectifying and deactivate at negative $V_m$. In both cases, the current decreases with an exponential time-course. Hence, the gating mechanisms resemble each other, but respond to opposite polarity. Moreover, the single-channel conductance is voltage-sensitive, yet the size is different (~25 and 60 pS, extrapolated to $V_m$ = 0 mV; see [18] and [27]).

What are the implications when VRAC and hemichannels are involved simultaneously? Provided that hemichannels are gating with negative voltage, this results in a shallow bell-shaped function $g_{ma} = f(V_m)$. This situation prevailed when Cx45 hemichannels were examined under osmotic stress (see Fig. 11). However, it could also be interpreted as behaviour of a single type of hemichannel (see [28]). Assuming that hemichannels are gating with positive voltage, this leads to an S-shaped function $g_{ma} = f(V_m)$ of negative slope. Hence, participation of two types of channels may not be obvious, even when using pharmacology. Interestingly, beyond this interaction, there is a possibility that VRAC and hemichannels are recruited sequentially under certain conditions. Based on the properties of these channels, we propose the following scheme. Hypotonic stress leads to cell swelling, opening of VRAC and subsequent release of nucleotides such as ATP$^2$ (see [18]). This in turn provokes opening of gap junction hemichannels [14] and closure of gap junction channels [25]. This pathway may be activated under physiological (e.g. cell proliferation, shear stress, secretion of fluid by glandular cells) as well as pathophysiological situations (e.g. ischaemia, congestive heart failure, hepatic cirrhosis). As suggested before (see [18]), ATP$^2$ released may then be involved in mediating autocrine/paracrine signaling via purinergic receptors.

Acknowledgements We thank D. Lüthy for technical assistance and B. Nilius, University of Leuven, Belgium, for suggesting the use of mibebradil. Transfectants were provided by K. Willecke, Institute of Genetics, University of Bonn, Germany. Supported by the Swiss National Science Foundation (31-55297.98, 31-67230.01).

References

5. OUTLOOK

The results obtained during this thesis offer several directions for future experiments. On the one hand, the identification and characterization of VRAC, the major interfering system when examining hemichannels, renders it possible to extend the studies on Cx45 hemichannels. An obvious continuation would be to explore e.g. the ionic selectivity of these channels to better understand the mechanism of channel permeation and filtering, or to examine the response of these channels to pCa_o and pCa_i, or analogously, to pH_o and pH_i to better understand the molecular mechanisms of chemical gating. Moreover, it would be interesting to find out if ATP$^{2-}$ depletion affects Cx45 hemichannels in a similar way as Cx43 hemichannels to find out if this is a specific or a general mechanism.

On the other hand, the methodological tools developed in this thesis may now be used to study the electrophysiological properties of hemichannels consisting of other connexins relevant for cardiovascular (e.g. Cx37, Cx40 and Cx43) and neuronal tissue (e.g. Cx26, Cx32, Cx36, Cx43 Cx45, Cx57). Such studies could also be extended to heteromeric hemichannels consisting of more than one kind of connexin (e.g. Cx40/Cx43, Cx40/Cx45 and Cx43/Cx45; these cells are available in this laboratory). Furthermore, it would be interesting to verify if the previously reported bell-shaped relationships $g_{hc} = f(V_m)$ of hemichannels (e.g. Cx30, Cx46, Cx50; Valiunas & Weingart, 2000) truly reflect hemichannel properties or superimposed sigmoidal relationships of a hemichannel and VRAC.
6. APPENDIX

6.1. Acknowledgement

This project would not have been possible without the support of many people. Many thanks to my adviser, Robert Weingart, who introduced me in the art of experimental science, read my numerous revisions and helped make sense of the confusion. Also thanks to my tutor, Hans Imboden, for his helpful input from several discussions. Thanks to Daniel Lüthi for technical assistance. Thanks to my colleagues in the Institute Marcel Egger, Thomas Desplantez, Lucinda Davies, Rolf Vogel and Cécile Choby. Thanks to Alexander Wildbolz and Christian Müller. And finally, special thanks to Luzia Enz, my parents Elsa and Paul Bader, my brothers Daniel and Andreas Bader and their families and my friends Luca Alberucci, Daniel Studer, Lukas Maurer, Zoula Papandreou and Fritz Bühlmann who endured this long process with me, always offering support and love.
7. CURRICULUM VITAE

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Education

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**Languages**

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**Publications**


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