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# Voltage sensitivity of gap junction currents in rat osteoblast-like cells

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**Abstract**

The dependence of macroscopic gap junctional conductance ($g_j$) upon transjunctional voltage ($V_j$) was examined in 39 paired osteoblast-like (OB) cells from primary cultures using the double whole cell patch clamp technique. OB cells were derived from calvarial explants of new-born rats. Instantaneous current–voltage ($I_j$–$V_j$) relationships of OB cell pairs ($n = 6$) were linear in the entire voltage range ($-150 \leq V_j \leq 150$ mV) examined. The steady-state $I_j$–$V_j$ relationship was non-linear for $V_j \geq \pm 60$ mV. The curve for the normalised steady-state junctional conductance–voltage relationship ($G_{ss}/G_0$–$V_j$) was bell-shaped, and was fitted with a two-state Boltzmann equation with a minimum conductance ($G_{min}$) of 0.2–0.3, and a half deactivation voltage ($V_o$) of $\pm 83$ mV. In two recordings unitary gap junction channel activity was observable. The linear $I$–$V$ relationships revealed a single channel conductance of $\sim 100$ pS. Application of parathyroid hormone ($10^{-8}$ M) had no effect on the voltage dependence nor the magnitude of macroscopic currents ($n = 7$).


*Keywords:* Osteoblast-like cell; Gap junction; Connexin43; Transjunctional voltage; Parathyroid hormone; (Rat)

## 1. Introduction

Gap junctions mediate the electrical coupling between neighbouring cells to allow cell-to-cell communication in various tissues. Two hexameric hemichannels, or connexons, form an intercellular channel between adjacent cells. Gap junction channels consist of a family of related proteins, called connexins, which contain highly conserved extracellular and transmembrane domains and divergent cytoplasmic regions. Connexin43 (Cx43) has been identified as the major gap junction protein in primary osteoblast-like (OB) cell cultures as well as in osteosarcoma cell lines [1–4]. Recently, expression of Cx43 and Cx45 in the osteoblastic cell lines ROS 17/2.8 and UMR 106-01, respectively, forming gap junctions with different molecular permeabilities, has been described [5]. In OB cells from primary cultures, mRNA for Cx45 is expressed in small amounts, compared to Cx43 [3,6]. The type of channels, homomeric, heterotypic and/or heteromeric are not known. Regardless, this finding suggests different types of signalling between cells, based on the type and number of gap junction channels present between two cells [7].

Electrical coupling between heart cells is essential

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for impulse conduction leading to co-ordinated contractions of the heart muscle. The voltage-dependent deactivation of gap junctions between heart cells has been extensively studied and been shown to have a time course of hundreds of milliseconds to seconds [8–12]. While the various connexins that can be present in various parts of the heart (Cx37/Cx40/Cx42/Cx43/Cx45) have different characteristics such as different voltage sensitivities [13,14], none is sufficiently voltage sensitive to restrict the rate of action potential conduction under physiological conditions [14,15].

OB cells are also electrically coupled. Shifting the membrane potential of one cell from −60 mV to −20 mV by current injections [16] leaves the coupling unchanged. OB cells also express some of the same connexins found in myocardial cells. The osteoblastic cell lines UMR 106-01 express Cx45 channels which have been found to be strongly voltage-dependent, whereas ROS 17/2.8 cell pairs express Cx43 channels with no voltage sensitivity [5]. We have studied the voltage-dependent junctional currents between primary cultured OB cells derived from calvarial fragments of new-born rats, which express both, Cx43 and Cx45 gap junction channels [3,6]. Double whole cell patch clamp [17] was used in the present study to examine the voltage dependence of gap junction channels between OB cells in primary culture. The observed behaviour is compared to the behaviour of Cx45 in mouse N2A neuroblastoma (N2A) cells and Cx43 in human smooth muscle cells and N2A cells [11,14,18]. We discuss the relevance of the findings in relation to action potential propagation in osteoblasts and second messenger intercellular communication.

## 2. Materials and methods

### 2.1. Cell culture

Calvarial fragments derived from new-born rats were explanted onto collagen-coated coverslips. They were maintained at 35° C in Hepes (20 mM) buffered minimum essential medium (88 vol%) supplemented with fetal calf serum (10 vol%), glutamine (200 mM; 1 vol%) and penicillin (5000 I.U./ml)/streptomycin (5000 I.U./ml; 1 vol%). All media components were purchased from ICN (USA). Nutrient medium was exchanged twice a week. Osteoblast-like (OB) cells were investigated 3 to 8 weeks after explantation.

### 2.2. Electrophysiological recordings

Cells were rinsed once in trypsin/EDTA (0.5 mg/ml; Sigma, USA). One ml fresh trypsin/EDTA was pipetted on to the cells and incubated for 2–3 min. To assess the trypsin activity cells were examined microscopically. Cells were gently scraped off the coverslips. When about 90% of the cells had lifted, 7 ml of media was added. The cell suspension was filled in a centrifuge tube and spun at 1800 rpm for 5 min. The supernatant was siphoned off. Cells were resuspended in the recording saline, plated on to 35 mm petri dishes (Falcon, USA) and allowed to settle 20 min before patching.

Junctional conductances of selected single calvarial cell pairs were determined using the dual whole cell patch clamp technique [17]. Patch pipettes were made from glass capillaries and filled with a solution containing (concentrations in mM) CsCl 185.0, EGTA 1.0, $NiCl_2$ 1.7, $CoCl_2$ 1.7, $MgCl_2$ 4.2, 4-aminopyridine (4-AP) 2.0 and Hepes 17 (pH 6.9) (resistances 1–2 M$\Omega$). Recordings were performed on cell pairs at room temperature in a saline composed of (concentrations in mM) CsCl 150, tetraethylammonium chloride (TEACl) 36, $CaCl_2$ 2.7, $NiCl_2$ 1.7, $CoCl_2$ 1.7, $MgCl_2$ 4.2, $ZnCl_2$ 0.2, 4-AP 2.1 and Hepes 17 (pH 7.1). All solutions were filtered (∅0.22 $\mu$m; Millipore, Bedford, MA, USA). In some experiments bovine parathyroid hormone (PTH; Sigma, USA) was added to the bath saline to a final concentration of $10^{-8}$ M.

After forming whole cell conditions, transjunctional potentials ($V_j$) were elicited by stepping the holding potential of one cell ($V_2$) from a common holding potential ($V_2 = V_1 = 0$ mV, when $V_1$ is the holding potential of the non-stepped cell) to a new value ($V_{2'}$). Since the Nernst potentials for all ions, except $Ca^{2+}$, across the junctional membrane and across the plasma membrane were 0 mV, both cells were held at 0 mV. Thus, in the recipient cell (cell held at 0 mV) the only unitary activity that could arise was from junctional membrane channels [15]. The polarity of current flow in the non-stepped cell is opposite to the stepped cell. Appropriate compensa-

tion for series resistance was always made. Input resistances ($R_i$) of the cells ranged from 50 to 500 M$\Omega$. Cells with $R_i < 50$ M$\Omega$ were not evaluated. Two Axopatch 200 amplifiers (Axon Instruments, Burlingame, CA, USA) were used to produce the driving voltage $V_2$ in cell 2 and to record resulting junctional currents from cell 1, respectively. Current signals in cell 1 and cell 2 were filtered at 1 kHz. Data were acquired with a PC-AT computer using pClamp software (Axon Instruments, Burlingame, CA, USA). A pulse program delivered rectangular voltage pulses. While one cell was held at 0 mV, the holding potential of the other cell was stepped to values, beginning with $-120$ mV for 300 ms. Then, voltage was switched to the opposite polarity for the same duration, inducing transjunctional potential ($V_j$) of $-120$ and 120 mV, respectively. Voltage steps were de- and increased in steps of 20 mV. In some experiments pulse series started from $-150$ to 150 mV. In the middle of each voltage pulse polarity was switched to the opposite direction to detect differences in gating properties. Voltage pulses were separated by a 5 s interval. A prepulse of 10 mV amplitude and 50 ms duration preceded each trial as a control of the integrity of the coupling.

The time course of the decay of transjunctional current ($I_j$) was examined at each $V_j$ above $\pm 60$ mV by fitting exponential functions to the decay phase of each $I_j$ trace [19].

### 2.3. Statistics

In order to test whether the parameters in control and in PTH containing solution were different, each of the data sets were fitted independently. In control, the parameters (mean $\pm$ standard deviation) were $V_0 = 92 \pm 11$ mV, $z = 2.7 \pm 1.0$ and $G_{min} = 0.31 \pm 0.15$. With PTH, the parameters were $V_0 = 76 \pm 8.3$ mV, $z = 2.89 \pm 1.44$ and $G_{min} = 0.33 \pm 0.13$. Although optimising these fits implies that the fit parameters are interdependent, we have tried to ascertain each parameter (e.g. $V_0$) change from control to PTH by the *t*-test [20]. For all three parameters, namely $V_0$, $z$ and $G_{min}$, the difference was not significant at the 5% level and even at the 10% level. Therefore the results in control and in PTH containing solution are asserted not to be significantly different.

## 3. Results

The voltage dependence of macroscopic junctional currents in primary cultures of calvarial cells (newborn rats) was investigated in 39 isolated cell pairs. The transjunctional current–voltage ($I_j$–$V_j$) relationship was analysed (i) in control saline ($n = 6$) and (ii) when cells were exposed to parathyroid hormone (PTH, $10^{-8}$ M) for 30–120 min ($n = 7$). In 26 cell pairs no voltage sensitive behaviour was observed. In 2 cell pairs with low conductances the activity of single gap junction channels were recorded.

### 3.1. Regulation of junctional currents by transjunctional potentials

Fig. 1A illustrates data from one experiment which is representative of junctional current in the non-stepped (recipient) cell. The 10 mV prepulse remains constant. With increasing transjunctional voltage the junctional current increases linearly up to $\sim \pm 60$ mV. At this voltage, $I_j$ showed a pronounced time- and $V_j$-dependent decay to steady-state values. Both, instantaneous ($\triangledown$) and steady-state ($\square$) current values were plotted as a function of $V_j$ (Fig. 1B). The instantaneous $I_j$–$V_j$ relationship was linear with a slope conductance ($g_j$) of 1.92 nS. Even at the largest $V_j$ tested, e.g. at $\pm 150$ mV, $I_j$ never reached zero. Similar results were obtained in five other cell pairs, where $g_j$ ranged from 0.4 to 56.7 nS.

### 3.2. Voltage-dependent deactivation of transjunctional currents

To determine the relationship between the steady-state conductance ($g_j$(ss)) and $V_j$, $g_j$(ss) was related to the instantaneous conductance ($g_j$(inst)) of each pulse, which was set to 1. The results of six experiments ($\bigcirc$) were pooled and are illustrated in Fig. 2. The normalised steady-state conductance ($G_{ss}/G_0$) was symmetrical around 0 mV, ranged from 0.8 to 1.0 for $-70 \leq V_j \geq 60$ mV and declined to a minimum value ($G_{min}$) of 0.2–0.3 at $\pm 150$ mV. A Boltzmann fit for the $G_{ss}/G_0$–$V_j$ relationship was performed for each polarity from data sets of six cell

pairs superfused with control saline by assuming two gates in series, by the formula:

$$(1 - G_{\min})\left(1 + \exp(-zV_o/50)\right)^2 \times \left(1/\left(1 + \exp(z(x - V_o)/25)\right)\right) \times \left(1/\left(1 + \exp(z(-x - V_o)/25)\right)\right) + G_{\min}$$

Here $G_{\min}$ is the normalised minimum conductance for large transjunctional voltages and $V_o$ is the half-activation voltage. $z$ is the number of equivalent gating charges that reflect the steepness of voltage activation. The parameters were $V_o = 83.12$ mV, $z = 2.29$ and $G_{\min} = 0.34$.

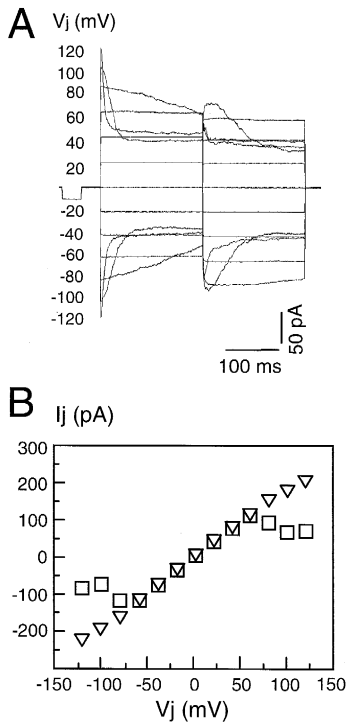


Fig. 1. Voltage dependence of gap junction currents elicited in rat osteoblast-like cells in vitro. (A) Several junctional current ($I_j$) recordings from the cell, which was held at 0 mV, while the holding potential of the other cell was altered systematically thereby inducing transjunctional voltage ($V_j$) pulses of different amplitude and polarity (left margin). In the middle of each voltage pulse polarity was switched to the opposite direction. A prepulse of 10 mV amplitude and 50 ms duration preceded each trial. $I_j$ reveals a time-dependent decay when ($V_j$) exceeds $\pm 60$ mV. (B) Instantaneous $I_j$ ($\triangledown$) and steady-state $I_j$ ($\square$) from the recordings above were plotted as a function of $V_j$. The straight line of the instantaneous $I_j$–$V_j$ relationship has a slope of 19.2 nS. The steady-state $I_j$–$V_j$ relationship deviates from linearity above $\pm 60$ mV and has a negative slope above $\pm 70$ mV.

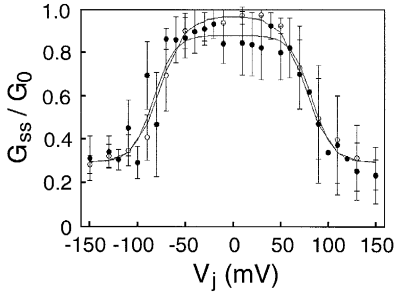


Fig. 2. Normalised steady-state junctional conductance ($G_{ss}/G_0$)–voltage ($V_j$) relationship calculated from six and seven cell pairs recorded in control saline ($\bigcirc$) and in parathyroid hormone ($10^{-8}$ M) containing solution ($\bullet$), respectively. Each point and bar represent mean $\pm$ S.E.M. $G_{ss}/G_0$ declines with increasing $V_j$ in either direction, while the steepest decay occurs at $V_j > \pm 70$ mV. The solid lines are the theoretical fits of the data assuming a two-state Boltzmann distribution with $V_o = 83$ mV and 77 mV and $G_{\min} = 0.33$ and 0.30, respectively.

We examined the effect of parathyroid hormone (PTH, $10^{-8}$ M), which is known to increase the concentration of intracellular cAMP on the voltage behaviour of OB cells. It is believed that this ultimately leads to phosphorylation of gap junction proteins in osteoblastic cells [4], which results in alterations in the gating and voltage sensitivity (cf. [21]). Using the same pulse protocol as described before, no difference was found in the voltage sensitivity of instantaneous and steady-state $I_j$ during exposure to PTH for 30–120 min, compared to controls. The range of junctional conductance for the PTH treated cells was 0.2 to 20 nS ($n = 7$). To determine the relationship between $g_j$(ss) and $V_j$, $g_j$(ss) was normalised to $g_j$(inst) of each pulse. The results of seven experiments performed in PTH-containing saline ($\bullet$) were pooled and are illustrated in Fig. 2. The normalised steady-state conductance ($G_{ss}/G_0$) was symmetrical around 0 mV, ranged from 0.8 to 1.0 for $-50 \leq V_j \geq 50$ mV and declined to a minimum value of 0.2–0.3 at $\pm 150$ mV. The $G_{ss}/G_0$–$V_j$ relationship was fitted by a two-state Boltzmann equation of the same form as that used for the data obtained in control solution. The parameters were $V_o = 77$ mV, $z = 1.96$ and $G_{\min} = 0.30$. These parameters are not significantly different from the control values ($t$-test, [20]).

### 3.3. Time course of deactivation

Fig. 3 shows examples of the single exponential fit of the junctional currents obtained from the cell pair shown in Fig. 1. The curves were fitted assuming that a model channel exists in either of two states, an open state with conductance $\gamma_j$ and a closed state of zero conductance, where the rate constants of channel opening and closing ($\alpha$ and $\beta$) are functions of the transjunctional voltage difference. The results with $G_{min} = 0.33$ in the form of rate constants are:

$$\alpha = 1.34 \exp\left(-2.325\left(V_j - 74\right)/25\right) \quad \text{and}$$

$$\beta = 1.34 \exp\left(3.05\left(V_j - 74\right)/25\right)$$

showing that $\sim 2$ and 3 equivalent charges are involved in channel opening and closing, respectively. The faint line represents the best fit with a single exponential to the data (Fig. 3). At the higher transjunctional voltages more than one time constant is needed to fit the data. However, the results of the first order fit, as embodied in the rate constants above, can be taken as an approximate way to encapsulate the results.

### 3.4. Single gap junction channel activity

Occasionally in control saline, gap junction channel currents could be resolved due to the voltage-dependent closure of gap junction channels leading to a decrease in the transjunctional conductance. The current–voltage ($I$–$V$) relationship of single gap junction channels was assessed by applying the same voltage protocol described before. Distinct channel openings and closures are shown in the recording from the cell held at 0 mV (Fig. 4). Due to noise, channel amplitudes were determined only between $\pm 50$ and $\pm 150$ mV. Fig. 4 illustrates the channel activity observed during application of pulses with the same protocol as that in Fig. 1. Discrete channel openings at the various voltages are seen in different traces. Due to uncoupling of cells during the experiment fewer channels with steps to higher potentials are active. All data yield a single channel conductance $\gamma_j$ of $\sim 100$ pS (cf. [22]).

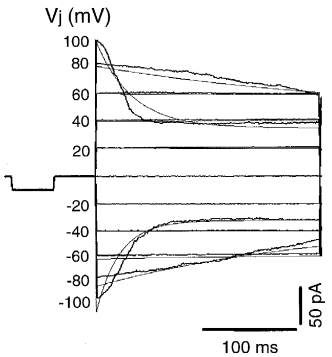


Fig. 3. Time course of decay of junctional current ($I_j$) measured from the cell shown in Fig. 1. The faint line represents the best fit with a single exponential of the decay phase of each $I_j$ trace recorded at different transjunctional potentials ($V_j$) (left margin). The calculated rate constants $\alpha$ and $\beta$ (for details, see text) indicate that two and three equivalent charges are involved in channel opening and closing, respectively.

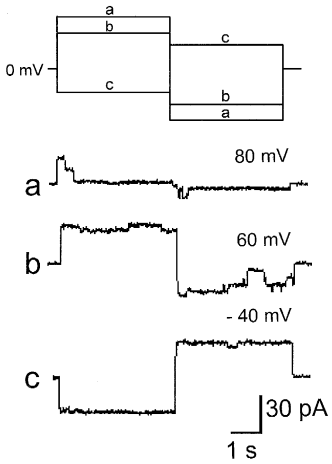


Fig. 4. Single channel activity of Connexin43 channels in osteoblast-like cells at different transjunctional potentials ($V_j$) induced by the pulse protocol. Discrete channel openings at various $V_j$ values are visible. $V_j$ is indicated in each trace (a–c) as obtained by the pulses shown in the upper part of the figure. In the process of the experiment the cells rapidly uncoupled. This is seen in the smaller current of trace a. Unitary conductance ($\gamma_j$) was calculated by dividing the amplitude of the unitary current by the amplitude of $V_j$ and was $\sim 100$ pS.

## 4. Discussion

In the present study, the dependence of gap junction currents from transjunctional potentials was determined between osteoblast-like (OB) cells in primary culture. This not homogeneous cell population

mainly secreted type I collagen and mineralised the extracellular matrix, which is typical for osteoblastic cells [2,23]. In OB cells, immunocytochemical studies and Northern blot analysis have shown Cx43 to be ubiquitous with lesser amounts of Cx45 present [2–4,6]. As gap junctions between OB are composed of the same connexins seen between cardiac myocytes, it was of interest to compare also their functional properties. The macroscopic data revealed linear slope conductances between 0.43 and 56 nS. Similar gap junctional conductances have been obtained from neonatal rat and adult guinea pig myocytes [8,13,24]. Also the single channel conductance of gap junctions in cultured OB cells is consistent with that reported by others for Cx43 channels [21,25].

Using standard pulse protocols, a symmetrical drop in $G_{ss}/G_0$ above $V_j$ values of $\pm 60$ mV was observed in 33% of cell pairs investigated. In the remaining 67% cell pairs, exhibiting similar transjunctional conductances ($g_j$), no voltage sensitivity was recorded. One explanation for the apparent lack of voltage sensitivity may point to cell pairs, which were connected by cytoplasmic bridges, indicating cell division processes. Another explanation for the variability of the voltage behaviour could be the inhomogeneity in primary cell culture. Third, in some cells the input resistance was low and the junctional conductance high ($> 40$ nS) precluding $V_j$-dependent behaviour due to reduced actual applied $V_j$ values [11,26]. In neonatal rats, a $V_j$-dependence of gap junction conductance was only observed when $g_j$ was $< 1$ nS [8]. In paired cardiac myocytes from adult heart, however, $g_j$ was found to be insensitive to transjunctional potentials [27,28]. Our findings indicate that the $V_j$ sensitivity was still observed in OB cell pairs with $g_j$ of 20–50 nS. The time course of deactivation, induced by applying transjunctional voltage pulses, was an exponential process, suggesting that deactivation is a complex phenomena, which may involve several components or more than one channel type. In all experiments, a voltage-insensitive residual conductance of 20–30% of the total conductance remained at $V_j$ values $> \pm 100$ mV. The residual conductance could arise from mode shifting (different channel opening and closing behaviour) or heterogeneous channel populations of Cx43 [18], or from voltage-insensitive substates [29,30] or from the presence of other channel types, e.g. Cx45.

Mammalian Cx43 gap junction channels are sensitive to the voltage difference across the junctional membrane exceeding $\pm 50$ mV and exhibit a residual conductance that is insensitive to even large $V_j$ [10,18,30,31]. The observation of a similar time- and voltage-dependent decline in junctional current, made in OB cells, was not seen in the osteoblastic ROS 17/2.8 cell pairs [5], which exclusively form Cx43 gap junctions, but in other systems, which contain only Cx43, voltage dependence was observed, $V_o \sim \pm 70$ mV [11]. In contrast, in UMR 106-01 cell pairs expressing Cx45, voltage sensitivity appeared when the transjunctional voltage difference exceeded $\pm 50$ mV [5]. This is more sensitive than $V_o$ of $\sim 80$ mV obtained here for controls and cells exposed to parathyroid hormone.

PTH had no significant effect on the voltage-dependent behaviour of gap junctions between OB cells compared to controls, which was assessed by theoretical fitting of the normalised $G_{ss}/G_0$–$V_j$ relationship assuming two gates in series. Junctional conductance or intercellular transfer of dye in different tissues has been shown to be increased by the presence of cytoplasmic cAMP or extracellular applied PTH [4,32,33]. PTH was shown to either increase or decrease the electric coupling in different OB cell pairs [34]. In addition, membrane potential changes had been induced by PTH in rat osteoblasts [35]. In this study, PTH stimulation of OB cells had no obvious effects on its voltage dependence or junctional conductance. A thinkable problem using trypsin for isolation of the cell pairs is the possible loss of cell surface receptors or channels. Damage of cell receptors was reported to occur at higher concentrations (10 mg/ml) [36], whereas at low concentrations of trypsin (1 mg/ml) no effects on cell surface structures were observed [37]. Thus, 0.5 mg/ml trypsin, used in our study, might have been too low to affect the PTH receptors in OB cell pairs. Another possibility might be that in OB cells regulation of gap junction channels differs from that of heart cells. It is possible that the effects of PTH might be functioning through enhanced recruitment of channels [1] rather than having a direct influence through a cAMP mediated phosphorylation. An additional possibility is significant washout of water soluble components via the patch pipette tip, which are important in the activation or regulation of receptor-mediated processes such as PTH stimulation.

Electrical coupling via gap junctions allows for rapid propagation of action potentials leading, e.g. to synchronous contraction of myocardial cells (cf. [38]). Gap junction channels might also play an important role in bone cell function regulation in a similar way. Stimulated and spontaneous membrane potential fluctuations of 5 to 15 mV amplitude had been observed in rat OB cells at membrane potentials of $-40$ mV and $-60$ mV, which may be conducted or passively spread via gap junctions from one cell to another [34]. In a minority of cultured OB cells, depolarisation waves have been elicited which resembled $Ca^{2+}$-mediated action potentials with amplitudes of up to 40 mV [34]. These observations may point to the significance of an information exchange system also in bone, which is not dependent on voltage changes in the physiological range.

Taken together, time and voltage dependence of gap junction channels in OB cells was similar to that found in heart cells and was not affected during a 30–120 min lasting exposure to PTH compared to controls, as Boltzmann fits revealed $V_o$ values, which were not significantly altered.

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