Analysis of the Presence and Physiological Relevance of Subconducting States of Connexin43-Derived Gap Junction Channels in Cultured Human Corporal Vascular Smooth Muscle Cells

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Abstract—Subconductance states are a commonly observed feature of gap junction channels. Their overt frequency and consistent appearance in both single and multichannel records have led to speculation that they might be of physiological importance in terms of altering the rate of small solute transfer from cell to cell. Among the connexin gene family, connexin43 (Cx43) is the most ubiquitous connexin that has been shown to generate subconductive states. Therefore, it was the explicit aim of this investigation to more fully evaluate the potential contribution of human Cx43-derived subconducting states to intercellular communication in cultured human corporal vascular smooth muscle cells. To determine the weight of subconductive states in our records, we analyzed amplitude histograms of multichannel and single-channel data during the application of transjunctional voltages larger than expected for physiological conditions but still smaller than transjunctional voltages known to induce lower conductive states (\(V_o \geq V_j\)). The data clearly indicated that the subconducting states occupy only a small fraction of the total channel open time. This was reflected by the fact that the average open probability for the subconductive state(s) determined from the 9 records analyzed was \(\approx 2\%\). Closer inspection of the data revealed that the frequency of subconductive states was actually higher than the frequency of the main state conductance. In summary, recording conditions sufficient for evaluation of the intrinsic gating characteristics of human Cx43-derived gap junction channels have been used. Under these conditions, our data clearly indicate that despite their greater frequency, the duration of subconductance events is so short relative to the main state duration as to render them physiologically insignificant. (Circ Res. 1999;84:797-803.)

Key Words: connexin ■ vascular smooth muscle cell ■ gap junction

Gap junction channels have a variety of properties that make them atypical ion channels. Chief among these are their long open time and correspondingly high open probability, as well as poor selectivity characteristics. Connexin43 (Cx43), the dominant gap junction protein in vascular smooth muscle, is no exception. Homotypic Cx43 channels show symmetric voltage dependence and are somewhat selective for cations over anions. Most connexins, including Cx43, have also been shown to allow the passage of small molecular weight probes (300 to 1200 Da\(^{-1}\)). Additionally, there is strong evidence that gap junction channels allow the diffusion of intracellular second messenger molecules such as Ca\(^{2+}\), cAMP, cGMP, and inositol 1,4,5-trisphosphate. These characteristics make the connexins important intercellular second messenger/ion pathways in many tissues. For example, it has already been demonstrated in vascular smooth muscle cells that Ca\(^{2+}\) can readily diffuse from cell to cell via channels composed of Cx43. In addition to their aforementioned permeability characteristics, the gating behavior of gap junction channels is also of interest. Cx43 has been well studied in this regard because it is one of the most ubiquitous connexins. Under steady-state conditions, estimates of mean open time (MOT) and mean closed time (MCT) have been made. A quantitative analysis of human Cx43 (hCx43) has yielded an MOT range of 0.43 to 5.25 s and an MCT range of 0.51 to 0.95 s. The study of hCx43 allowed determination of the open probability (\(P_o\)), which was found to range from 0.5 to 0.95. This indicates that hCx43 remains open most of the time. Extrapolation of MOT and MCT to predicted values at \(V_j=0\) mV yields a \(P_o\) equal to 0.81.

All channels display, to varying degrees, subconducting states or sublevels. Gap junctions are also no exception in this regard. Cx43, for example, can display a main state of 90 to 100 pS with a sublevel of 30 pS in 150 mmol/L of salt. A subconducting state of 60 pS has also been reported.
These different conducting levels (sublevels) have been correlated with phosphorylation levels for Cx43. 13

The occurrence of subconductance states in gap junction channels raises two immediate questions. First, are the selectivity characteristics of the substates the same as or different from the main state? Second, do the substates play a role in the voltage-dependent processes of gap junctions? Recent studies11 showed that one subconductance state for Cx43 was more restrictive than the main state by 50%, but the data also indicated that, as documented for the main state, the subconducting state was still permeable to both cations and anions. Further, it has been argued that the substates of Cx43 are less voltage dependent than the main state and thus comprise a large portion of the steady-state conductance observed with large \( V_j \) gradients. 14 For hCx37 this is clearly the case. 15

A third question arises as well. Is the frequency or dwell time of the subconducting states sufficient to be of significance with regard to affecting junctional conductance or permeation of intercellular messengers when \( V_j \) is in the voltage-independent portion of the \( G_j/V_j \) relationship (ie, \( V_j \) steps<\( V_j \))? This last case questions whether the substate conductances occur with sufficient frequency and duration to affect intercellular communication under in situ conditions, and this issue was addressed by the present study. To this end, we studied Cx43-derived gap junction channel activity in the well-characterized cultured human corporal smooth muscle cells. 5,6,12

**Materials and Methods**

Records analyzed in the present study arose from the database published in Brink et al. 6 The data were generated by using dual whole-cell patch-clamp techniques as described elsewhere in detail. 6 Briefly, for all records analyzed, the bathing solution was composed of (in mmol/L) CsCl 165, TEACl 30, CoCl2 1, NiCl2 1, MgCl2 1, CaCl2 2, HEPES 10, aminopyridine 1, TTX 0.01, and ZnCl2 0.5 (pH 7.0). The pipette solution was the same except for the following: 0.1 mmol/L CaCl2 was used, 0.6 mmol/L EGTA was added, and there was no ZnCl2; the pH was 6.8. In 2 experiments, CsCl was replaced by NaCl with no detectable difference in channel activity. That is, qualitatively similar substate events were present in both the cesium and sodium solutions. Multichannel records were analyzed using the procedures of Ramanan and Brink16 and Ramanan et al. 17 Amplitude histograms were fit using fit parameters outlined in Ramanan and Brink16 and Veenstra et al. 18 For experiments (n=3) designed to examine Cx43 substates under conditions previously documented to promote their formation,13 we used 2 mmol/L of the membrane permeant cAMP analogue 8-bromo-cAMP (Sigma). Records were obtained after 5 minutes and 30 minutes of exposure. All data were low-pass filtered at 5 kHz and stored on videotape via Neurocorder. To digitize the data, they were played back and low-pass filtered at 0.5 kHz and acquired through a 16-bit A/D converter into a Next computer. The sampling rate was 0.180 ms.

**Results**

The occurrence of substates in gap junction channels is well documented, and the current tracings provided in Figures 1 and 2 highlight this fact. Figure 1 shows representative records from both cells of a pair, clearly indicating, by virtue of the presence of paired junction current signals of opposite polarity, the junctional origin of the substates. More specifically, the recordings in Figure 1 illustrate the equal but opposite rule for gap junction channels. These records show that in addition to main state transitions, substate transitions are observable in both records, removing any doubt that the lower conductance transitions represent another population of channels. Subsequently, the records from the cell held at zero or near zero potential are shown. Under the ionic conditions used, the equilibrium potential for all nonjunctional channels is zero, with the exception of calcium; therefore, no contaminating nonjunctional channel activity can occur. In Figure 2, the top and middle panels show typical transjunctional current recordings, which show channel closures and subconducting states for hCx43. The top panel shows the subconductance state in more detail. The duration of the substate is quite short relative to the closure of the main state and is very short relative to the duration of the open state. Rarely are
subconducting states observed arising from the ground state to the subconducting level for Cx43 gap junction channels. More typical is the incomplete closure or lower conductive state shown in Figure 2. The transjunctional voltage (Vj) in these records was 40 mV.

Figure 3 is an all-points histogram of a larger portion of the records shown in Figure 2. The histogram constitutes a total of 300 seconds of data. As illustrated, several distinct fitting parameters were used. Clearly, the record is best interpreted as a single channel with no substate. The unitary conductance used in this fit was 105 pS for the main state, and on the basis of the transitions shown in Figure 2, a subconducting state of 65 pS was presumed. Five distinct cases are shown in Figure 3. In Figure 3A, a single 105-pS channel is assumed with no weighting for a substate. P_o for the main state is 85%. In Figure 3B, a single 105-pS channel with a 65-pS substate is assumed where P_o for the main state is 0.85 and the substate is 0.03. A small decline in the peak of the closed state fit is the result. In Figure 3C, the P_o for the main state was dropped to 0.80, and a subconductance of 65 pS was given a P_o of 0.06. The closed peak fit is only slightly decreased, but a significant increase in the height between the open and closed states is observable. In Figure 3D, P_o for the main state was further decreased to 0.725 to allow a substate weighting of 0.125 or 12.5%. The closed peak fit remains close to the fit shown in Figure 3A, but a notable peak or increase in area exists between the open and closed peaks, which represents the subconductance weight. The dashed line fit of Figure 3A appears to fit the data the best. In this case, no subconductive state was needed.

In summary, despite the documented presence of the subconductance states, which generally ranged from ~30 to 65 pS, the weighted amplitude histogram shows little evidence for their contribution to the overall open time of the channel. The reason for the apparent discrepancy between the recordings (Figures 1 and 2) documenting that the substate exists, and the fits in the amplitude histogram, which cannot account for the presence of the substate, is related to the fact that the despite their frequency, their duration renders them insignificant in the weighting of the amplitude histogram.

In light of these considerations, we attempted fitting the data under yet another condition. In Figure 3E, the dashed line represents the predicted amplitude histogram if 2 independent channels, one of 105 pS and one of 65 pS, are assumed to be in the patch. As illustrated, the data are
Figure 3. Amplitude histograms of a 300-s record, a corresponding portion of which is shown in Figure 1. The same amplitude histogram was computer fit in the absence and presence of substate weighting (computer fits are shown by dashed line). For all fits, the main state conductance was 105 pS, and subconductive state on the basis of Figure 1 was determined to be 65 pS. A, Histogram plus fit where $P_o = 0.85$, no substate weighting. B, Same as panel A, only substate weighting of 0.03. Note decline in fit to closed peak. C, Histogram with fit in which $P_o$ of the main state is 0.80; substate 0.06. The fit is not as good as in panel A or panel B. D, $P_o = 0.725$ for the main state and 0.125 for the substate. The fit and data clearly deviate. E, Histogram and fit assuming 2 independent channels, one of 65 pS and one of 105 pS.
inconsistent with the 65-pS channel representing an independent channel, or the histogram would have the form of that shown by the dashed line in Figure 3E.15

Figure 4 shows another example of the data in which the amplitude histogram clearly shows subconductive states. In this case, the data were taken from a 50-s record. To fit this data set, 2 different $P_o$ values had to be used for each of the 2 channels in the patch. This is not an uncommon feature for multichannel gap junction records.$^{17,18}$ To fit the amplitude histogram, a subconductive state weighting of 0.06 was required for both channels (dashed line). In these records, the unitary conductance of the main state was 115 pS and that of the subconductive state was 45 pS. The entire recording is shown in Figure 5. The middle panel shows the whole recording and the top and bottom panels show representative main state openings and closures, as well as transitions to the subconductive state.

All the histogramic data are summarized in the Table (n=9 experiments). In 5 of these experiments, no weighting for subconductive states was necessary. In 4 of the data sets, weighting for subconductive states was necessary to fit the data. The average weighting for those 4 data sets was 0.042 or 4.2%. Taking all the records into account yielded an average of 0.02 or 2% weighting for the subconductive state(s). To further examine the potential contribution of substates to intercellular communication, we evaluated the effects of phosphorylating treatments known to promote the formation of the subconducting states$^{13}$ on our junctional recordings. Thus, the effects of 2 mmol/L 8-bromo-cAMP were tested in 2 additional experiments (see Materials and Methods). There was no detectable change in the weight of
The data shown illustrate that the gating of the hCx43 subconductance states is such that the number of (partial) transitions per unit of time can be equal to or exceed the number of complete open-close transitions. Nonetheless, the observed substates constitute a very small fraction of the open time for the hCx43 gap junction channel when transjunctional voltage is less than the $V_c$ of Cx43, $\sim 80$ mV. This phenomenon is best illustrated by the amplitude histograms, which are a simple and effective method for determination of the total open time for the main open state, as well as any detectable subconductance states. As illustrated, the data indicate a maximum weight of the open peak(s) of $\sim 6\%$ for the observed substates (Figures 3 and 4; Table). However, more often than not (Table), the subconductance states constitute an even smaller percentage of the open portion of the amplitude histogram.

In light of these results, it seems relevant to ask the following question: On the basis of the current in vitro data, is there any role for gap junction channel subconductive states in the functioning of multicellular tissues that are reliant on intercellular communication for coordination of their respective responses? In short, the current data argue against such a possibility. Specifically, it is not a question of whether or not the biophysical characteristics of the substates themselves would confer any degree of permselectivity but rather an issue of whether or not the substates stay open for sufficiently long periods of time to alter the passage of enough solutes, relative to the main state, to be physiologically relevant. That is, if subconductance states are to have
meaningful effects on cell-to-cell transfer of small solutes, then the open probability of the subconductance states would be predicted to occupy a larger fraction of the open dwell time than we estimate, on the basis of our current observations. As such, our observations indicate that the permelectivity of hCx43 subconductive states is secondary to the dwell time with regard to any possible effects on solute transit for Cx43.

In short, the main issue is not the selectivity but rather the relative contribution of the substate to the total open time. For example, the average mean open time for the main state of hCx43 has been estimated to be 1.4 s. If the subconductance state or states represent 2% of the average mean open time, then the average mean open time or dwell time for the subconductive states is estimated to be $\approx 28$ ms. Clearly, such a substate frequency and duration are not sufficient to alter the transit of solutes from cell to cell relative to the expected contribution of the main state. To further evaluate the potential contribution of subconducting states to intercellular communication, we examined the effects of phosphorylating treatments known to promote their formation. In 2 experiments, treatment of cells with 8-bromo-cAMP had no detectable effect on the weight of the subconductive states in the amplitude histogram. As such, it would seem that under the experimental conditions used in the present study, subconducting states play little, if any, role in modulating intercellular communication between human vascular smooth muscle cells.

In conclusion, if similar substate phenomena do indeed exist in the in situ environment, then substates most certainly would serve a similarly minor role in the modulation of intercellular communication in multicellular tissues. Clearly, this supposition awaits verification in vivo.

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References
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