Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels

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INTRODUCTION

The gap junction proteins connexin32 and connexin43 are phosphoproteins, whereas connexin26 is not (e.g., 1, 2). Treatment of cells with phosphorylating agents (most notably the tumor-promoting phorbol esters, here abbreviated TPA) result in various actions on gap junctional conductance (g_j) when applied to cells of different tissues (e.g., 3). These different effects may be attributable to the different complements of gap junction proteins expressed by the different cell types, to tissue-specific as distinct from connexin-specific effects, or may represent effects on distinct synthetic or processing stages in the expression pathway (4). To evaluate the effects of phosphorylating and dephosphorylating treatments on gating properties (voltage dependence and unitary conductance) of gap junctions formed of a known connexin, we transfected a communication-deficient cell line (SKHep1) with cDNA encoding human connexin43 (hCx43;5). Furthermore, we compared the gating properties of the wild type channels with those truncated so as to remove the phosphorylation sites (6). These studies indicate that unitary conductance and voltage dependent kinetics (but not equilibrium) properties of the human connexin43 gap junction channel are affected by phosphorylation, and that the presence of the carboxyl terminal phosphorylation sites is not required for functional expression of junctional channels.

RESULTS AND DISCUSSION

SKHep1 cells were cotransfected with vectors containing the selectable marker *neo* and full-length rat and human connexin43; stable transfectants were isolated. Northern and Western blots, and g_j and dye transfer measurements verified abundant connexin43 expression (5). Western blots of the human connexin43 transfectants with connexin43-specific antibodies revealed multiple isoforms of this gap junction protein, with a promi-

nent band at ~41 kDa and a doublet at ~43–45 kDa (5). Immunoprecipitation of connexin43 from ^{32}P-labelled cells revealed that the upper bands were phosphorylated but the 41 kDa band was not (unpublished results with Drs. J. C. Sáez and E. L. Hertzberg), confirming previous studies by others (7–9).

Single channel studies on transfectants, in which unitary junctional currents were recorded after halothane exposure (which by itself does not alter unitary conductance γ_j;10) revealed two populations of channels, with γ_j ~60 and 90 pS (Fig. 1). In frequency histograms of channel transitions under normal conditions, the larger channel event occurred about twice as often as the smaller channel.

Because these cells exhibited multiple isoforms of connexin43 that were related to phosphorylation state, it seemed plausible that the different channel sizes might also represent different phosphorylation states of the junctional channel. To test this hypothesis, cell pairs were treated with the phosphorylating agents TPA (12-O-tetradecanoylphorbol-13-acetate; 0.1 μM: a tumor promoting phorbol ester that activates kinase c), 8 Br-cAMP (8-bromo cyclic adenosine monophosphate; 1 mM: a membrane permeant cAMP analogue that activates kinase a), forskolin (0.5 mM: which activates adenylyl cyclase), and okadaic acid (300 nM: which inhibits endogenous phosphoprotein phosphatases and increased hCx43 phosphorylation).

Each of these phosphorylating treatments produced marked effects on the distribution of γ_j values, where the frequency of the larger events was much reduced compared to that of the smaller event. After frequency histograms were constructed, the fraction of total events of each size were calculated and the ratio of 60–90 pS events were compared to that of the control condition. Relative to control, the 60/90 ratio was increased about two-fold by forskolin, ~5-fold by cAMP and by TPA and ~20-fold after okadaic acid treatment.

Effects of dephosphorylating agents were also examinated (staurosporine, a protein kinase inhibitor, and uni- or bilateral exposure of the cell interior to 14 μg/ml of alkaline phosphatase (AP), through the patch pi-
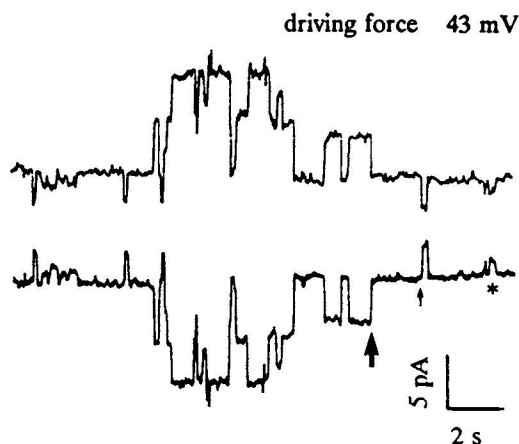
driving force   43 mV

5 pA

2 s

FIGURE 1   Single-channel recordings from SKHep1 cells stably transfected with human connexin43 exhibit two conductance levels. Channels of two sizes due to connexin43 are apparent (90 and 60 pS, larger and smaller arrows), as are occasional junctional channels (*asterisk*) of the size of the endogenous junctional channels expressed to a low degree in the parental cell line.

A   Gj

o control
▲ phosphatase

Transjunctional voltage (mv)

B

o control
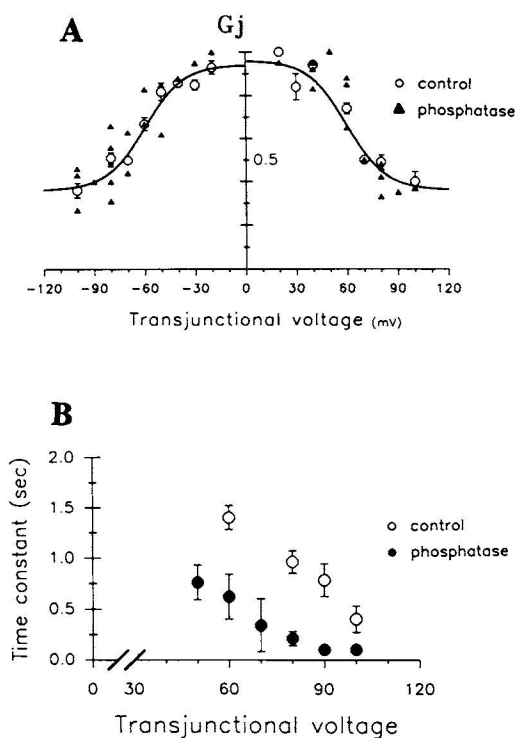● phosphatase

Transjunctional voltage

FIGURE 2   Voltage dependence of connexin43 channels under control conditions and after phosphatase treatment. (*A*) Boltzmann relation for the steady-state dependence of normalized junctional conductance (G_j) on transjunctional voltage in cells transfected with human connexin43. For experiments before (*open circles with SE bars*) and after phosphatase treatment (*filled triangles,* individual experiments), there is no apparent change in the parameters that describe the voltage dependence (V_0, the voltage at which the voltage dependent component of the conductance is reduced by half, is ~60 mV for each group; and g_{min} is ~40% with and without phosphatase). (*B*) Voltage dependence of the time constant of relaxation of junctional current before (*open circles*) and after (*filled circles*) phosphatase treatment. Bars correspond to SEs for at least 3 τ values for each voltage for each treatment.

pettes). Compared to the control condition, staurosporine approximately doubled the frequency of the larger events. Even more dramatic effects on the distribution of γ_j values were seen with patch electrodes containing low concentrations of AP (heated to 100°C for 1 min to decrease proteolytic activity), with virtually all of the events being of the larger size.

Steady-state and kinetic properties of voltage dependence were compared before and after phosphatase treatment; although steady-state values were unchanged (Fig. 2A), the time constant was markedly decreased over the entire voltage range (Fig. 2 B). For pulses of a single polarity, the two-state reaction scheme predicts that the normalized voltage sensitive equilibrium conductance $(g_\infty - g_{min})/(g_{max} - g_{min})] = \alpha/(\alpha + \beta)$ where α and β are the opening and closing rate constants, respectively; whereas the time constant of the relaxation is given by $\tau = 1/(\alpha + \beta)$. Evaluation of rate constants indicates an approximately threefold increase in both α and β. Thus, our results suggest that dephosphorylation of the channel protein results in symmetric changes in voltage sensitivity of the transitions between open and closed states.

To determine whether channel phosphorylation was directly involved in these effects, and to localize the phosphorylation sites within the connexin43 sequence, we compared these results with properties of connexin43 constructs lacking phosphorylation sites (6). Voltage dependence of mutants expressing truncated connexin43 (phosphorylation sites removed) was similar to the parental channels; γ_j values were single-valued for

each, consistent with the hypothesis that the multiple conductance states of the wild type human connexin43 channels correspond to the multiple phosphorylation isoforms.

We conclude that phosphorylation of hCx43 reduces the unitary conductance of gap junction channels and reduces the values of the rate constants governing its voltage dependence and that these phosphorylation sites are not required for insertion or assembly of human connexin43 into functional channels.

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