In adult mammalian muscle, the chloride conductance ($g_{Cl}$) is by far the largest component conductance at resting potential (Bryant & Morales-Aguilera, 1971; Rüdel & Senges, 1972; Palade & Barchi, 1977; Kwiecinski et al. 1984). Chloride conductance is 4–5 times larger than the resting potassium conductance and is thus a major determinant of the rheobasic current necessary to generate an action potential (Adrian & Bryant, 1974). Regulatory pathways modulating muscle Cl$^{-}$ channels, therefore, play a major role in adjusting the excitability of the muscle fibre membrane. Brinkmeier & Jockusch (1987) demonstrated that treatment with phorbol esters induced repetitive myotonic discharges in mouse muscle fibres by diminishing sarcolemmal $g_{Cl}$, and concluded that phosphorylation of muscle chloride channels by protein kinase C (PKC) was responsible for this effect. Further experiments with amphibian and mammalian muscles (Bryant & Conte-Camerino, 1991; Tricarico et al. 1991; Tricarico et al. 1993) indicated that phosphorylation by protein kinase C is a specific regulatory mechanism of mammalian muscle chloride channels.

6. The two forms of congenital myotonia, the dominant Thomsen's disease and Becker's recessive generalized myotonia, both characterized by muscle stiffness caused by the generation of repetitive action potentials induced by a diminished $g_{Cl}$, are due to genetic alteration of hClC-1 (Koch et al., 1995).

**Regulation of the human skeletal muscle chloride channel hClC-1 by protein kinase C**

Angela Rosenbohm*, Reinhardt Rüdel* and Christoph Fahlke†

*Abteilung Allgemeine Physiologie, Universität Ulm, Oberer Eselsberg, D-89069 Ulm, Germany and †Departments of Medicine and Pharmacology, Vanderbilt, University School of Medicine, 21st and Garland Avenues, Nashville, TN 37232, USA

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1. The regulation of a recombinant human muscle chloride channel, hClC-1, by protein kinase C (PKC) was investigated in human embryonic kidney (HEK 293) cells.

2. External application of 4β-phorbol esters (4β-PMA) reduced the instantaneous whole-cell current amplitude over the entire voltage range tested. This effect was abolished when the cells were intracellularly perfused with a specific protein kinase C inhibitor, chelerythine. Inactive 4α-phorbolesters did not affect the chloride currents. We conclude that the effect of 4β-phorbol esters is mediated by protein kinase C (PKC).

3. Activation of PKC resulted in changes in macroscopic current kinetics. The time course of current deactivation determined in the presence and absence of 4β-phorbol esters could be fitted with the sum of two exponentials and a constant value. In the presence of phorbol esters, the fast time constants and the minimum value of the fraction of non-deactivating current were increased, whereas the voltage dependence of all fractional current amplitudes remained unchanged. PKC-induced phosphorylation had only small effects on the voltage dependence of the relative open probability and the maximum absolute open probability was unaffected by treatment with 4β-PMA, as shown by non-stationary noise analysis.

4. The kinetic changes indicate that phosphorylation alters functional properties of active channels. Since the absolute open probability is not reduced, the observed macroscopic current reduction implies alterations of the ion permeation process.

5. Phosphorylation by PKC appears to affect ion transfer and gating processes. It is postulated that the phosphorylation site may be located at the cytoplasmic vestibule face of the pore.
et al. 1992; George et al. 1993). The hCIC-1 channel was shown to consist of two subunits (Fahlke et al. 1997), and expression of a dimeric construct linking two hCIC-1 coding regions in a single open reading frame gives rise to the expression of chloride currents indistinguishable from results obtained from cells transfected with the corresponding monomeric construct (Fahlke et al. 1997). These results taken together provide strong evidence that the muscle chloride channel in native tissue, as well as in heterologous expression systems, is built by two hCIC-1 units without contribution of any other CIC-isofoms.

The aim of this work was to investigate if activation of protein kinase C affects recombinant hCIC-1 channels in heterologous expression systems and, if so, to obtain clues on the responsible mechanism. The Results section is divided into two parts: in the first part we show that 4â-phorbol esters reduce macroscopic hCIC-1 current amplitudes in heterologous expression systems specifically by activating protein kinase C-mediated phosphorylation. In the second part, the kinetic changes induced by 4â-phorbol esters in mammalian cells will be used for an analysis of the mechanism of the macroscopic current reduction.

A preliminary report of these results has appeared (Rosenbohm et al. 1995)

METHODS

Whole-cell recording in stably transfected HEK 293 cells

The human embryonic kidney (HEK 293) cells stably expressing hCIC-1 channels were generated and cultured as previously described (Fahlke et al. 1995). Transient expression was performed with tsA201 cells (a simian virus (SV40) large T antigen expressing derivative of the HEK 293 cell line) as recently described (Fahlke et al. 1997). Chloride currents were recorded in different recording modes of the patch-clamp technique (Hamill et al. 1981) using an EPC-7 (List, Darmstadt, Germany) or an Axopatch 200A (Axon Instruments, Foster City, CA, USA) amplifier. Pipettes with resistance of 0–5–1.5 MΩ were pulled from borosilicate glass. For excised patch recordings, pipette tips were coated with dental wax (The Hygienic Corporation, Akron, OH, USA) to reduce pipette background noise. In all recordings, more than 60 % of the series resistance was compensated by an analog procedure.

Application of phorbol esters

Stock solutions of the phorbol esters 4â-phorbol-12-myristate-13-acetate (4â-PMA; Sigma, Deisenhofen, Germany), and 4â-phorbol-12,13-didecanoate (4â-PDD; Sigma) in DMSO were prepared at a concentration of 10 mg ml−1 and diluted with external solution to the desired final concentration of 1 μM 4â-PMA and 1 μM 4â-PDD. For the non-stationary noise analysis, cells were pretreated with 1 μM 4â-PMA added to the culture medium for at least 20 min. In experiments, in which protein kinase C was specifically inhibited, 1 μM chelerythrine (LC Laboratories, San Diego, USA) was added to the pipette solution.

Data analysis

Amplitude and time course of the chloride currents were analysed using a combination of pCLAMP and SigmaPlot (Jandel Scientific, San Rafael, CA, USA) programs. In order to average current amplitudes obtained from different cells (Fig. 1), instantaneous and steady-state current amplitudes were normalized to the instantaneous current amplitude determined at a test potential of −145 mV in each cell. Steady-state activation curves were obtained by plotting the instantaneous current amplitudes at a fixed potential of −125 mV after a 5 s prepulse at a potential between −125 mV and +75 mV vs. the prepulse potential. Dividing each data point by the maximum of the instantaneous current observed in the experiment yielded the voltage dependence of the relative steady-state open probability (Fahlke et al. 1996). The time course of chloride current deactivation was fitted with a sum of two exponentials and a time-independent value:

\[ I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + d, \]

where \( I(t) \) represents current amplitude, \( a_1, a_2, \) and \( d \) amplitude factors, and \( \tau_1, \) fast and \( \tau_2, \) slow time constants.

For each test potential, relative coefficients \( A_1, A_2, \) and \( C \) were derived by dividing the absolute values by the peak current amplitude \( I_{\text{max}} \), i.e., \( A_1 = a_1/I_{\text{max}}, \) \( A_2 = a_2/I_{\text{max}} \) and \( C = D/I_{\text{max}} \) (Fahlke et al. 1996). For the linear transformation of fractional current amplitudes before and after PKC action (see Results, E↵ect of PKC activation on deactivation parameters), scaling factors were chosen to obtain identity of the transformed fractional current amplitude before PKC activation with the experimentally observed fractional current amplitude after PKC activation at the most negative and the most positive test potential. For this purpose a set of two linear equations \( F(V_{\text{phosphorylated}}) = a \times F(V_{\text{control}}) + b, \) where \( V_0 \) is either −145 or −55 mV was solved to obtain the scaling factors \( a \) and \( b \). Rate constants for the fast deactivation process at negative potentials were calculated using the equations:

\[ k_{\text{in}} = (1 - E)/\tau_1 \] and \[ k_{\text{off}} = E/\tau_2, \]

where \( E \) represents the amplitude of the fractional current \( C \) at the most negative applied potential (Fahlke et al. 1996). The voltage dependence of the instantaneous whole-cell chloride conductance, \( g_{\text{Cl}} \), was calculated from:

\[ g(V) = [I(V + 5 \text{ mV}) - I(V - 5 \text{ mV})]/10 \text{ mV}, \]

and the equation:

\[ g(V) = g_{\text{max}}/(1 + \exp((V - V_0)/k_V)) \]

was fitted to the experimental data.

For non-stationary noise analysis, current responses to 120 subsequent identical test steps to −145 mV preceded by a 250 ms prepulse to +75 mV were recorded. Mean current (\( I(0) \)) and mean variance (\( \sigma^2 \)) were calculated using standard formulae. Baseline
variance ($\sigma^2$) was measured at 0 mV. The number of ion channels per patch ($N$) and the unitary current ($I$) were obtained by fitting:

$$\sigma^2 = i I - \frac{i^2}{N} + \sigma^2_o.$$  (1)

The number of ion channels ($N$) and the unitary conductance ($i$) were used to calculate absolute open probabilities from current recordings by:

$$p(t) = \frac{I(t)}{Ni}.$$  (2)

All results are given as means ± s.e.m. Student's $t$ test was applied for statistical evaluation and level of significance are given as *5% probability and **1% probability.

**RESULTS**

**Effects of phorbol esters on recombinant hClC-1 channels**

**Experiments with HEK 293 cells**

Application of 1 $\mu$m 4$\beta$-phorbol-12-myristate-13-acetate (4$\beta$-PMA) caused a reduction of the amplitude as well as a change on the kinetics of the expressed chloride currents (Fig. 1). The control currents (Fig. 1A) illustrate the characteristic properties of hClC-1, inward rectification of the instantaneous current, and bi-exponential deactivation at negative potentials which is incomplete even at very negative potentials (Fahlke et al. 1995, 1996). Addition of

![Figure 1](image)

**Figure 1. Chloride currents in the absence and presence of 1 $\mu$m 4$\beta$-PMA, recorded from HEK 293 cells at pH 7.0**

A, responses of the current to voltage steps in 40 mV increments from a holding potential of −30 mV and through the range −145 and +15 mV. B, same cell and same pulse programme, 5 min after addition of 1 $\mu$m 4$\beta$-PMA. C, mean instantaneous current–voltage relationship before (○) and after (□) addition of 1 $\mu$m 4$\beta$-PMA ($n=4$). D, mean steady-state current–voltage relationship determined from the same two sets of traces. E, mean instantaneous current–voltage relationship obtained 20 min after the addition of 1 $\mu$m of the inactive analogue 4$\alpha$-PDD ($n=4$, different cells). F, mean steady-state current–voltage relationship determined from the same two sets of traces as used for E. In C–F instantaneous and steady-state current amplitudes were normalized to the instantaneous current amplitude determined at a test potential of −145 mV in the same cell.
1 μM 4β-PMA produced alterations of macroscopic current characteristics. Figure 1B shows current recordings from the same cell illustrated in Fig. 1A after modification by 4β-PMA. The instantaneous current amplitude was reduced over the entire tested voltage range (−145 to +15 mV) and the deactivation process was slowed. Moreover, deactivation was less complete so that the typical crossing over of current traces seen in the controls with hyperpolarization did not occur (Fig. 1B). In the absence of 4β-phorbol esters, current amplitudes as well as current kinetics were unchanged during a similar observation period.

The voltage dependences of the instantaneous currents determined before, and 20 min after application of 4β-PMA is shown in Fig. 1C (closed and open symbols, respectively). In these experiments, 4β-PMA reduced the current amplitudes at −145 mV to 55 ± 5% (n = 4) of control. The chloride conductance, calculated as the slope of the line fitted to the responses to test pulses between −145 and −85 mV, was decreased to 53%. The steady-state current–voltage relationship lost its characteristic dip around −80 mV and increased monotonically with more positive membrane potential (Fig. 1D). Exposure of cells to phorbol esters for larger periods (up to 1 h) did not result in a more pronounced current reduction.

The reversal potential of the currents was not affected by 4β-PMA. This was observed with three different transmembrane chloride gradients. With [Cl]e/[Cl]i = 500 mm/154 mm the reversal potential was 3.5 ± 3.5 mV before and −0.0 ± 3.1 mV after application of 1 μM 4β-PMA (n = 9). We repeated the experiments with two other pipette solutions and observed comparable levels of current reduction under these conditions. With [Cl]i = 24 mm, the reversal potential was −29.6 ± 6.2 mV before and −28.4 ± 6.6 mV (n = 7) after 4β-PMA modification. For [Cl]i = 5 mm, we obtained corresponding values of −67.5 ± 2.6 and −67.5 ± 1.4 mV (n = 4). Therefore, 4β-PMA does not affect the selectivity of hClC-1 channels. Furthermore, changes in current amplitude are not due to contamination with currents carried by ions other than chloride.

The specific property of 4β-phorbol esters is to activate PKC (Castagna et al. 1982). Nevertheless, a variety of non-specific effects of these agents have been reported (Hockberger et al. 1989). To show that the reported effects of 4β-PMA are due to activation of PKC and subsequent phosphorylation of hClC-1 channels, we used two different approaches. First, we compared the effects of corresponding 4α- and 4β-phorbol esters (Castagna et al. 1982). Application of 4α-phorbol-12,13-didecanoate did not cause any of the changes induced by the 4β-phorbol ester (Fig. 1E and F).

Next, we measured effects of 4β-PMA while protein kinase C is specifically blocked by chelerythrine (Herbert et al. 1990), added to the pipette solution at a concentration of 1 μM. Chloride currents were first monitored for a period of 15 min, then 4β-PMA was added and the time course of possible changes of chloride currents was observed. Using this experimental protocol, we observed a starting value of −8.3 ± 0.8 nA (instantaneous current amplitude at −145 mV), before addition of 4β-PMA −8.7 ± 1.2 nA, and −8.5 ± 0.6 nA (n = 3) 20 min after 4β-PMA addition. The 4β-PMA effect was therefore prevented by intracellular perfusion with chelerythrine. Moreover, we did not observe any increase of the current amplitude during intracellular perfusion with chelerythrine, suggesting that there is no tonic inhibition of hClC-1 channels in HEK 293 cells by PKC under normal conditions.

These experiments illustrate that the effect of 4β-PMA on chloride channels in HEK 293 cells stably expressing hClC-1 is mediated by activation of protein kinase C.

Effect of PKC activation on hClC-1 gating and permeation

We next undertook an analysis of gating and permeation properties before and after addition of 4β-PMA to describe further functional alterations of hClC-1 channels by PKC-induced phosphorylation. We tested PKC effects on gating properties at three different pH values. Intracellular pH modifies certain properties of hClC-1 channel gating (Fahlke et al. 1996). Experiments in which the effect of PKC activation was investigated at different pH, promised to provide insights into which gating features (Fahlke et al. 1996) are affected by phosphorylation. At all tested pH values, PKC induced changes of hClC-1 current amplitude and kinetics. The degree of reduction of the chloride current is influenced by pHi value: with pHi at 6·0 the amplitude was only reduced to 81 ± 19% and with pHi at 8·5 it was reduced to 41 ± 20% of control (n = 4); (20 min after application of 4β PMA). However, the different degree of current reduction does not interfere with the interpretation of our results, pHi is only used for modifying gating mechanisms in hClC-1.

Effect of PKC activation on deactivation parameters

The time course of current deactivation of the chloride currents upon hyperpolarizing voltage steps can be fitted with a sum of two exponentials and a time-independent value. Two time constants of deactivation derived from these fits are almost independent of the membrane potential, but the fractional amplitudes of the three different deactivating current components are strongly voltage dependent (Fahlke et al. 1995, 1996). We recently proposed a gating model of hClC-1 with a set of two voltage sensors, the conformation of which defines three kinetic states, and a cytoplasmic gate mediating opening and closing transitions by directly occluding the pore (Fahlke et al. 1996). In the framework of this model, the kinetic states correspond to three distinct pore conformations characterized by different dissociation constants for permeating anions (Fahlke et al. 1997) as well as for the proposed cytoplasmic gate (Fahlke et al. 1996). Deactivation upon hyperpolarization is caused by an open channel block by this cytoplasmic gate and can be described as a first order process (Fahlke et al. 1996).

The fractional current amplitudes of the fast (A1, Fig. 2A) and the non-deactivating (C, Fig. 2C) components as well as
the fast time constants ($\tau_1$, Fig. 2D) were changed by activation of PKC whereas the fractional current amplitude of the slowly deactivating component ($A_1$, Fig. 2B) and the slow time constants ($\tau_2$, data not shown) were unaffected. For the fractional amplitudes $A_1$ and $C$, a linear transformation (see Methods) can convert values after PKC activation to control values (results of this transformation are shown as crosses in Fig. 2A and C). This demonstrates that the voltage dependence of the fractional current amplitude is unchanged in phosphorylated channels. The observed changes in channel gating can be fully described by assuming that only the rate constants governing binding and unbinding of the cytoplasmic gate to the internal channel opening are affected by protein phosphorylation. We used the fast time constants and the limiting value of the non-deactivating fraction of chloride channels at a test potential of $-145$ mV to calculate two rate constants, $k_{on}$ and $k_{off}$ (Fahlke et al. 1996). This calculation was done for three different pH values both before and after PKC activation (Fig. 2E and F). PKC activation causes a decrease of $k_{on}$ at pH 7·0 and 8·5. Rate constant $k_{off}$ is increased by PKC activation at all tested pH values.

We conclude that phosphorylation does not interact with voltage-dependent gating processes but affects the voltage-independent opening and closing transitions.

The PKC-mediated effect on steady-state activation
Steady-state activation curves were constructed by plotting the instantaneous current amplitudes at a fixed test potential vs. the variable prepotential (see Methods). In

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**Figure 2. Effects of 1 μM 4β-PMA on the kinetic parameters of chloride current deactivation**

$A$, $B$ and $C$, plots of $A_1$, $A_µ$ and $C$, the relative current amplitudes of the fast and slow deactivating and the time-independent components of the chloride currents, respectively, vs. the membrane potential. For calculation of these coefficients see Methods. Values are means ± s.e.m. from 8 experiments performed at pH 8·5. $D$, time constant $\tau_1$ (see Methods) of fast deactivation vs. membrane potential. Values are means ± s.e.m., $n = 4$ cells at pH 8·5. $E$ and $F$, rate constants $k_{on}$ (transition from open to closed state of chloride channel) and $k_{off}$ (transition from closed to open state) vs. pH values. Before (●) and after (±), addition of 4β-PMA. Results of a linear transformation (+; see Methods) from data after modification. Levels of significance are given as * 5% probability and ** 1% probability.
Fig. 3, experiments on cells with a pH of 7·0 are shown. When the test pulse was preceded by a positive prepotential, activation of PKC caused a reduction of the chloride current amplitude values (Fig. 3A and B), whereas the small current amplitudes recorded after negative prepulses were little affected by PKC. This corresponds to the previously mentioned less complete deactivation at negative potentials (Fig. 1A and B).

To compare the voltage dependence of activation obtained in the absence and presence of 4β-PMA, the data points of Fig. 3B were divided by the corresponding peak current amplitude, to obtain plots of the relative open probabilities (Fig. 3C). Neither the midpoints of activation (before 4β-PMA: −37·6 ± 0·9 mV, after: −36·5 ± 1·2 mV, n = 4), nor the slopes obtained at the midpoints (27·8 ± 1·4 mV, and 28·8 ± 1·4 mV, n = 4) are changed by addition of 4β-PMA. Similar results were obtained for two other pH values. Whereas the minimum relative open probability was increased by 4β-PMA for all tested pH values, the midpoint of the activation curve (at pH 6·0, 11·1 ± 4·8 and 11·8 ± 4·6 mV, n = 4; at pH 8·5, −20·1 ± 4·0 and −17·9 ± 1·9 mV, n = 4) and the slopes (at pH 6·0, 25·2 ± 3·6 and 29·8 ± 4·0 mV and at pH 8·5, 25·4 ± 1·0 and 23·9 ± 1·9 mV, n = 4) are unchanged.

PKC-mediated phosphorylation does not affect the absolute open probability

The quantitative description of the steady-state activation curves as well as of the deactivation time course showed that PKC-mediated phosphorylation does not affect the voltage dependence of gating in hClC-1 channels. Nevertheless, these measurements only provide data about the relative open probability and do not exclude major changes of the absolute open probability. We used non-stationary noise analysis to obtain values for absolute open probabilities. Current recordings were performed on inside-out or outside-out patches excised either from control cells or from cells pretreated with 1 μM 4β-PMA for at least 20 min. Figure 4A and B shows averaged current traces from 120 consecutive voltage steps to −145 mV after a 250 ms lasting prepulse to +75 mV recorded from two inside-out patches excised from tsA201 cells transiently expressing hClC-1. Whereas the left column illustrates control data, the right represents recordings from channels pretreated with 1 μM 4β-PMA. The corresponding time course of the current variance is illustrated in Fig. 4C and D.

Assuming a single conductance state, the dependence of the variance on the current amplitude can be described (Sigworth, 1980). Fitting this parabola (eqn (1)) to the variance vs. current relationship, one can obtain the number of active channels and the unitary current of this single conductance state (Fig. 4E and F). From the unitary current and the number of channels per patch, the time course of current deactivation can be transferred into a decline of absolute open probability (eqn (2), Fig. 4G and H). In 4β-PMA pretreated cells, the absolute open probability is unchanged compared with control data (absolute open probability at +75 mV: 0·66 ± 0·06 (n = 3), control value, 0·65 ± 0·05 (n = 2), after 4β-PMA treatment). Similar results were obtained for two patches from pretreated and three from control cells.

The finding that the absolute open probability at +75 mV is unchanged, together with the increase of the relative open probability at more negative potentials (Fig. 3), demonstrates that the absolute open probabilities of phosphorylated channels are not smaller than those of control channels. The observed decrease of the macroscopic current amplitude is therefore not due to alterations of channel gating and so must be due to alterations of the ion conduction pathway. This conclusion can unfortunately not be directly demonstrated, since the unitary conductance of hClC-1 channels is too small to allow a direct observation of single channels (Puch et al. 1994). Moreover, the ionic pore undergoes voltage-dependent conformational changes which affect the affinities of anionic binding sites within the ion conduction pathway (Fahlke et al. 1997b). These results indicate that hClC-1 has multiple open states with distinct...
conductance values preventing an accurate assessment of single channel conductances using noise-analysis methods.

**Inward rectification of chloride currents**

The conductance of hClC-1, represented by the slope of instantaneous current–voltage relationships (Fig. 1C and E), decreases as the membrane potential becomes more positive, i.e. hClC-1 is an inward rectifier (Fig. 1C). The molecular mechanism of this inward rectification is not fully understood, but the voltage dependence of the conductance of a whole HEK 293 cell transfected with hClC-1 can be well described with a single Boltzmann equation:

\[
g(V) = \frac{g_{\text{max}}}{1 + \exp\left(\frac{V - V_0}{k_v}\right)}.
\]

(Fahlke et al. 1995). Addition of 4β-PMA caused \(g_{\text{max}}\) to decrease to 47 ± 16% at pH 7.0 (n = 13), and to 34 ± 18% at pH 8.5 (n = 7). This agrees with the changes of the instantaneous current amplitudes measured for these

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**Figure 4. Non-stationary noise analysis on currents through control and 4β-PMA pretreated hClC-1 channels**

A and B, mean current amplitudes obtained from excised inside-out patches from control (A) or from cells pretreated with 4β-PMA (B). A, voltage steps to −145 mV was preceded by 250 ms prepulse to +75 mV. C and D, corresponding mean variance. E and F, variance–mean current plot from the data shown in A, B, C and D. Continuous lines are fits to eqn (1). The fitted parameters are as follows: \(I = 0.33\) pA, \(N = 14\) 850 for the control patch (E); \(I = 0.23\) pA, \(N = 10\) 660 for the 4β-PMA pretreated channels (F). G and H, time course of the absolute open probability for recordings shown in A and B. Mean current traces were divided by the number of active channels and the unitary conductance obtained from fits shown in E and F.
two pH values. Both Boltzmann parameters, \( V_{o} \) and \( k_{v} \) were characterized by a high variance and were not significantly different in the absence and presence of 4\( \beta \)-PMA. The respective \( V_{o} \) values were \(-74.5 \pm 20.9 \) and \(-62.7 \pm 20.9 \) mV for pH 7.0; and \(-53.3 \pm 12.0 \) and \(-63.6 \pm 9.1 \) mV for pH 8.5. The corresponding slope factors \( k_{v} \) were 50.6 \pm 6.1 and 50.3 \pm 6.5 mV for pH 7.0; and 47.5 \pm 9.5 and 36.1 \pm 13.1 \) mV for pH 8.5.

**DISCUSSION**

Protein kinase C-mediated phosphorylation affects channel function

4\( \beta \)-phorbol ester decreases macroscopic hClC-1 currents in a characteristic manner. The effect can be prevented by intracellular perfusion with chelerythrine, a specific blocker of protein kinase C (Herbert et al. 1990). Moreover, 4\( \alpha \)-stereoisomers do not affect chloride currents. These results indicate that phosphorylation by protein kinase C affects heterologously expressed hClC-1 channels. The effects of PKC-mediated phosphorylation on channel function, i.e. altered gating and permeation properties, suggest that the phosphorylation site is hClC-1, or another closely associated subunit, but this has not yet been directly demonstrated. There are ten potential sites for PKC-mediated phosphorylation in the hClC-1 sequence. Site-directed mutagenesis will allow a definitive test whether the actual phosphorylation site is within the hClC-1 sequence.

Comparison with native muscle

In intact mouse muscle, \( g_{o} \) decreased within 30 min to less than 10% of control when 4\( \beta \)-phorbol-12,13-diacetate was given at a concentration of 1-6 \( \mu \)M (Brinkmeier & Jockusch, 1987). In intact goat muscle, the Cl\(^{-}\) current was reduced to 24% upon application of 2 \( \mu \)M 4\( \beta \)-phorbol-12,13-dibutyrate (Bryant & Conte-Camerino 1991). Tricario et al. (1991) reported a reduction to 33% with rat muscle fibres.

In our experiments, the reduction caused by 1 \( \mu \)M 4\( \beta \)-phorbol-12-myristate-13-acetate was to no less than 45% and we are therefore unable to fully reproduce the effect of protein kinase C on native muscle chloride channels in heterologous expression systems. The reasons for this difference are unclear. A possible explanation is the difference of recording techniques. In experiments with native muscle, the \( g_{o} \) is extracted from passive membrane properties. The conductance measured in the absence of extracellular chloride is subtracted from the corresponding value obtained in the presence of extracellular chloride to obtain the pure \( g_{o} \). This procedure depends on the hypothesis that the intracellular chloride concentration approaches zero in the absence of extracellular chloride. Although this assumption appears valid in control muscle fibres exhibiting a large \( g_{o} \), intracellular chloride accumulation through active processes is likely to be more important in muscle fibres with decreased \( g_{o} \). In this case, the intracellular permeant anion concentration differs from zero, and the subtraction procedure thus underestimates \( g_{o} \). Moreover, methane sulphonate, the anion with which chloride is normally substituted in these experiments, was shown to be permeant to hClC-1 (Fahlke et al. 1997a). Besides these methodological problems, the initial reduction of the chloride conductance by PKC may cause additional changes of the intracellular milieu in native muscle. These alterations, like changes of the intracellular pH, effects on [Cl\(^{-}\)], etc., may further reduce the \( g_{o} \).

**Mechanism of current reduction by activation of PKC**

A decrease of macroscopic current amplitudes can arise from three different changes of single channel properties (Siegelbaum & Tsien, 1983; Imaimov & Benes, 1995) a decline in the number of functional channels, a reduction of the single channel open probability and a decrease in the single channel current amplitude. The observed changes of the deactivation time course (Figs 1 and 5), namely an increased steady-state current component and increased fast deactivation time constant, rule out the possibility that a decrease in number of functional channels is the only effect of protein kinase C-mediated phosphorylation. Phosphorylation affects functionally active channels. We could also exclude a shift of the chloride reversal potential or an alteration of the voltage dependence of activation as causes for the observed macroscopic current reduction. Since we cannot detect changes in the absolute open probability measured with noise analysis, we conclude that the ion permeation pathway is affected. The current reduction after activation of PKC is therefore most probably due to an alteration of ion conduction process. Because of the small unitary conductance (Pusch et al. 1994) and the occurrence of multiple, quantitatively different open states (Fahlke et al. 1996, 1997b), this cannot be demonstrated more directly i.e. by single channel or noise analysis measurements.

**Speculations about structure-function relationship**

The unaffected voltage dependence of fractional current amplitudes (Fig. 2) and the unchanged slope of the steady-state activation curve (Fig. 3) after PKC stimulation lead us to conclude that there is no interaction between the phosphorylation site and voltage-sensing mechanisms. Upon PKC stimulation, the fast deactivation time constant was slowed and the fraction of the non-deactivating current component was increased. Modelling this as a first order process, we were able to show that phosphorylation affects both rate constants and thus interacts with the negatively charged internal gate (Fahlke et al. 1996). The affinity of this blocking particle to its acceptor is thus decreased by phosphorylation of a nearby site.

A likely explanation for the observed effects is that the phosphorylation site is located in the inner vestibule of the ion pore. This hypothesis would also account for the presumed effect on the ion conduction process. The identification of the actual phosphorylation sites will help localizing structural determinants of the acceptor and of the proposed cytoplasmic gate and thus increase our understanding of ion permeation and gating in voltage-gated chloride channels.


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Corresponding author

Ch. Fahlke: S-3223 MCN, Vanderbilt University Medical Center, 21st Avenue South and Garland, Nashville, TN 37232-2372, USA.

Email: christoph.fahlke@mcmail.vanderbilt.edu