Functional Study of CLC-1 Mutants Expressed in *Xenopus* Oocytes Reveals that a C-terminal Region Thr891-Ser892-Thr893 is Responsible for the Effects of Protein Kinase C Activator

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Key Words

CLC-1 • Chloride channel • Mutant • Phosphorylation site • Protein kinase C • Phorbol ester

Abstract

CLC-1 plays an important part in the maintenance of membrane potential in the mammalian skeletal muscle. To investigate the phosphorylation sites responsible for the effect of PKC (protein kinase C) activator, we constructed 21 different CLC-1 mutants with mutations at predicted phosphorylation sites for PKC. The functional experiments were performed on both wild-type and mutant proteins (17 point mutants and 4 double mutants) expressed in *Xenopus* oocytes with two-electrode voltage-clamp recording. PMA (12-myristate 13-acetate), a PKC activator, caused a right shift of half-maximum activation potential ($V_{1/2}$) significantly in the wild-type (from -42.9 ± 4.4 to -13.7 ± 1.7 mV; $n = 8$, $P < 0.05$) and most of the single mutants except the S892P (from -39.5 ± 4.5 to -35.7 ± 5.7 mV; $n = 6$) and S892D (from -10.2 ± 4.9 to -9.6 ± 3.5 mV; $n = 4$). S892D, a mutant mimicking PKC-mediated phosphorylation at position 892, can also mimic the effect of wild-type treated with PMA in $V_{1/2}$ value (-10.2 ± 4.9 mV vs -13.7 ± 1.7 mV, $n = 4 - 8$).

However, S892A still had a significant response to PMA indicating that other sites responsible for PMA might exist. Thus double mutants are generated for the following analysis. The $V_{1/2}$ of double mutants, T891A/S892A, S892A/T893A and T891A/T893A, show no significant difference between before and after PMA treatment. We hypothesize that this structural modification results in the observed alteration of the gating properties of CLC-1 by PMA. In summary, our observations show that a C-terminal region Thr891-Ser892-Thr893, at least in part, responsible for the effect of PMA on CLC-1.

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Introduction

CLC-1, a chloride channel in mammalian skeletal muscle membrane, plays an important part in the regulation of the membrane excitability [1]. The chloride conductance contributes for ~80% of the total resting conductance in mammalian muscle fibres, and thereby stabilizes resting membrane potential in skeletal muscle. Reductions of chloride conductance of skeletal muscle

by chloride channel inhibitors [2-3] destabilize the resting potential and lead to a repetitive firing abnormality (also known as myotonia) [4]. Mutations in the *CLIC-1* gene are responsible for human myotonia congenita that can be either autosomal recessive or autosomal dominant [5]. It has been reported that chloride conductance in skeletal muscle is decreased by phorbol esters, presumably through activation of protein kinase C (PKC) [6]. PKC inhibitor chelerythrine was able to increase the chloride conductance in soleus muscle, suggesting that partial PKC-dependent activation of *CLIC-1* channels in basal conditions may contribute to the lower chloride conductance in slow-twitch muscles [7]. Furthermore, the staurosporine (a PKC blocker) treatment alone in dissociated fibers culture for 1-2 days indicating chloride conductance is downregulated by a mechanism involving protein phosphorylation [8]. It was also shown that a recombinant human *CLIC-1* expressed in HEK293 cells is regulated by PKC activators [9-10]. All of these studies demonstrated that the inhibitory effect of phorbol esters on chloride channel in skeletal muscle is mediated, at least in part via the PKC-dependent pathway. However, the exact position of its phosphorylation sites of *CLIC-1* is still unknown. The discovery of the gene structure and function of mammalian *CLIC-1* has been described previously [11, 12]. In human, *CLIC-1* protein is composed of 988-amino acid, including a 398-amino acid carboxyl-terminal tail that resides in the cytoplasm and contains two CBS (cystathionine- β -synthase) domains [13]. Functional analysis of human *CLIC-1* channel showed the PKC appears to affect gating processes and it is suggested that the phosphorylation site may be located at the cytoplasmic region [9]. PKC preferentially phosphorylates serine or threonine residues that are close to a C-terminal basic residue [14-15]. Bioinformatic tools can help to predict possible phosphorylation sites in the C-terminal region of *CLIC-1*. However, their results must be confirmed through functional experiments. Through the computational prediction, there are about twenty potential phosphorylation sites located at this C-terminal domain; each potential site is recognized by PKC. Therefore we designed and constructed over twenty mutant plasmids by using the methods of site-direct mutagenesis. Wild-type and mutant *CLIC-1* channels were heterologously expressed in *Xenopus laevis* oocytes and characterized by measuring whole cell currents with two-microelectrode voltage clamp techniques. Our results explored the residues Thr891-Ser892-Thr893 of *CLIC-1* are candidates for the effects of PKC activator.

Materials and Methods

PKC phosphorylation sites prediction and site-directed mutagenesis

We use phosphorylation site prediction Netphos server [16-17] for the prediction of potential PKC phosphorylation sites within the C-terminus of *CLIC-1*. Based on the prediction, we constructed 21 mutant plasmids of human *CLIC-1*, and then expressed each of the *CLIC-1* mutants in *Xenopus* oocytes for measurement of the chloride current. For each predicted site was replaced with an appropriate amino acid residue by site-directed mutagenesis method as previous described [18-19]. The mutations of nucleic acids of *CLIC-1* mutants and the mutagenic primers used in this study are shown in Table 1. In brief, the corresponding mutations were introduced into human wild-type *CLIC-1* cDNA in the pTLN *Xenopus* oocytes expression vector by recombinant PCR using Pfu turbo DNA polymerase (QuickChange Site-Directed Mutagenesis kit; Stratagene). DNA from these putative mutant colonies was sequenced to confirm the site-directed mutation and to exclude the error sequence.

In vitro transcription

The *in vitro* transcription of *CLIC-1* cRNA was performed using a mMESAGE mMACHINE SP6 Kit (Ambion) according to the manufacturer's protocol. Plasmids were linearized with *HpaI*, and capped cRNA was transcribed using SP6 RNA polymerase. The cRNA was purified and resuspended in nuclease-free water at a concentration of 1 mg/ml and stored frozen at -80°C in aliquots. The concentration of each RNA was determined by UV spectrophotometry.

Oocytes preparation and microinjection

Female *Xenopus laevis* frogs were anaesthetized by immersion in 0.15% Tricaine (Sigma-Aldrich, USA) which dissolved in 0.1% sodium bicarbonate solution. The ovarian lobe was then surgically removed from the abdominal cavity through a small incision and placed in modified Barth's solution (MBS) containing the following (in mM): 90 NaCl, 1 KCl, 2.4 NaHCO₃, 0.82 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, and 10 Hepes-Tris, pH = 7.6. Small pieces of ovarian lobe were dissected out and gently shaken at room temperature for 1 hour in MBS solution supplemented with collagenase type 1 (1-2 mg/ml) followed by five to six washes in MBS. The defolliculated oocytes (Stage V or VI) were then incubated and kept overnight at 17 °C before injection with mRNA. Only healthy-looking oocytes of V-VI stages were selected for microinjection. Wild-type and the mutants cRNA were microinjected into defolliculated *Xenopus* oocytes (5-10 ng/cell) using a Nanoject microinjector (Drummond, USA). Injected oocytes were kept in sterile MBS at 17-18 °C for 2-3 days before electrophysiological measurements were performed. All experiments conformed to guidelines of the ethical use of animals of the Animal Care Committee of Chung Shan Medical University.

Mutants	Nucleotides	Primers
T782R	2345CG-F 2345CG-R	5'-cagagctcgcgccagaaagaagaaaca-3' 5'-tgttttcttcttctggggcgagctctg-3'
T782A	2344AG-F 2344AG-R	5'-gcagagctcgcgccgaaagaagaaaca-3' 5'-tgttttcttcttctggggcgagctctg-3'
S886R	2658CA-F 2658CA-R	5'-cctcccttgccagattccggaacacac-3' 5'-gtcgtgttccggaatctggcaagggagg-3'
S892P	2674TC-F 2674TC-R	5'-tcggaacacgactccaactcgaagagt-3' 5'-actcttcgagttggagtcgttccgga-3'
S896R	2688TA-F 2688TA-R	5'-tcaactcgaagagaaacggggcacctcc-3' 5'-ggaggtgccccggttctcttcgagttga-3'
S682A	2044TG-F 2044TG-R	5'-cgcggaagtggcggagctgcctta-3' 5'-taaggcagctccgcaacttccgcg-3'
S720A	2158TG-F 2158TG-R	5'-acgaagatctcgtcgcaagagcga-3' 5'-tcgctcttccagcagatcttctg-3'
S740A	2218TG-F 2218TG-R	5'-ctgccccctctgccccagagagcc-3' 5'-ggctcttctggggcagagggcgag-3'
T782A	2392TG-F 2392TG-R	5'-tggataacatggcacctgaagagat-3' 5'-atctcttcaggtgccatgttatcca-3'
T890A	2668AG-F 2668AG-R	5'-gcttcggaacgcgacttcaactcg-3' 5'-cgagttgaagtcgcttccggaag-3'
T891A	2671AG-F 2671AG-R	5'-tcggaacacggcttcaactcgaaa-3' 5'-tttcgagttgaagccgtgttccgga-3'
S892A	2674TG-F 2674TG-R	5'-cggaacacgactgcaactcgaagaa-3' 5'-tcttccgagttgcagtcgttccg-3'
T893A	2677AG-F 2677AG-R	5'-aacacgacttcagctcgaagagtag-3' 5'-gtactcttcgagctgaagtcgtgtt-3'
S902A	2704TG-F 2704TG-R	5'-gggcacctccagcttctgcagagaa-3' 5'-ttcttcgagaagctggaggtgtccc-3'
T929A	2785AG-F 2785AG-R	5'-cctccccagaggccctgtgccatc-3' 5'-gatggcacaggggacctgtggagg-3'
S933A	2797TG-F 2797TG-R	5'-ccccctgtgccagctcttccccaga-3' 5'-tctggggaaggagctggcacagggg-3'
T891A S892A	T891A S892A-F T891A S892A-R	5'-ggaacacggctgcaactcgaagag-3' 5'-ctcttccgagttgcagccgtgttcc-3'
T891A T893A	T891A T893A-F T891A T893A-R	5'-aacacggcttcagctcgaagagtag-3' 5'-tactcttcgagctgaagccgtgtt-3'
S892A T893A	S892A T893A-F S892A T893A-R	5'-aacacgacttcagctcgaagagtag-3' 5'-tactcttcgagctgcagtcgtgtt-3'
S892D	2675CA-F 2675CA-R 2676AC-F 2676AC-R	5'-ggaacacgactgaaactcgaagag-3' 5'-ctcttcgagttcagtcgtgttcc-3' 5'-gaacacgactgacactcgaagag-3' 5'-actcttcgagtcagtcgtgttcc-3'

Table 1. The sequences of the primers used for site-directed mutagenesis in this study. F: forward primer; R: reverse primer.

Electrophysiology

Wild type and mutant CIC-1 channels were heterologously expressed in *Xenopus* oocytes and the currents were recorded by using a two-electrode voltage-clamp amplifier (AxoClamp-

Fig. 1. The effects of PMA on wild-type and mutants of S682A, S720A, S740A and T782A. Human CIC-1 chloride currents (left side) before and after application of PMA (2 μ M) were obtained by two-electrode voltage clamp from oocytes expressing wild-type (WT, A) and mutants (B-E). The right part of the figure shows the plotting graphs of chloride current activation curves. The effects of a significant right shift of half-voltage activation potential ($V_{1/2}$) induced by PMA in mutations of S682A, S720A, S740A and T782A are similar to that of wild-type.

2B, Axon Instruments). All electrodes were pulled from borosilicate glass capillaries GC150T4 (Warner Instruments) on a vertical electrode puller (PP-830, Narishige) and filled with 3M KCl solution (0.5 to 2 M Ω). One of the electrodes was used as a voltage recording (connected to an HS-2A x 1 LU headstage) and the other electrode was used for current recording (connected to an HS-2A x10 MGU headstage). The oocytes were placed in a 2 ml recording chamber during experiments and the membrane chloride current was measured in ND 96 solution consisting of (mM): 96 NaCl, 1 KCl, 1 MgCl₂, 1 CaCl₂, 5 Hepes; pH was 7.4. All recordings of membrane currents were carried out at room temperature (22-26 $^{\circ}$ C). Membrane currents were digitized at 5 kHz through a Digidata 1322A A/D converter, acquired, and analyzed using pClamp 9.0 software. The apparent open probability was determined by a

step protocol, whereby the oocytes were clamped at -100 mV and test potentials ranging from -140 to +100 mV in 20 mV increments followed by a constant tail pulse to -80 mV.

Calculations and data analysis

Peak tail currents at this voltage were fitted using a Boltzmann distribution as follows,

$I(V_m) = I_{(\max)} / \{1 - \exp[(V_{1/2} - V_m)/k]\}$, where $I_{(\max)}$ is the maximal tail current, V_m is the membrane potential, $V_{1/2}$ is the half-maximum activation voltage, and k is the slope factor. Data are presented as means $\pm$ SE. Statistical significance was determined using ANOVA and Student's *t*-test. *P* values of < 0.05 were taken to indicate statistical significance.

Results

Most of the single mutants had a significant response to phorbol 12-myristate 13-acetate (PMA)

Wild-type and 21 independent variants of CIC-1 were heterologously expressed in *Xenopus* oocytes to investigate the effect of PMA (a PKC activator) on CIC-1 channels. The wild-type CIC-1 currents show a significant change in the channels' gating properties after addition of PMA (2 μ M) (Fig. 1). The half-maximum activation voltage ($V_{1/2}$) was significantly right-shifted from -42.9 ± 4.4 to -13.7 ± 1.7 mV ($n = 8$, $P < 0.05$) before and after application of PMA, respectively. There are no significant differences in $V_{1/2}$ values between the mutants (S682A, S740A and T782A) and wild-type. The activation curves of each of these mutants (S682A, S720A, S740A and T782A) are sensitive to the treatment with PMA, which result in a significant right-shift of $V_{1/2}$ to more positive potentials respectively (on the right side of Fig. 1B to Fig. 1E). The left sides of Fig. 1A to 1E show typical current traces through CIC-1 channel in response to voltage pulse. Here we show only some examples of current traces with single mutants. The summarized data of other single mutants with a significant response to PMA are shown in Fig. 5.

The effects of PMA on T890A, T891A, S892A, and S892P

The C-terminal tail of CIC-1 contains a region with four successive residues of Thr-Thr-Ser-Thr at position 890 to 893, which are the potential targets for PKC. Point mutation of these potential residues was created for the study. There were no significant differences in $V_{1/2}$ among T890A (Fig. 2A), T891A (Fig. 2B), S892A (Fig. 2C), S892P (Fig. 2D), T893A and wild-type (see also Fig. 5). T890A, T891A, S892A and T893A can also give

Fig. 2. Effects of PMA on the mutants of T890A, T891A, T892A and S892P. On the left side of the figure shows the typical current traces before (upper) and after (lower) PMA treatment in T890A (A), T891A (B), T892A (C) and T892P (D) respectively. The $V_{1/2}$ of activation curves (right side) were significantly shifted to more positive potentials by PMA in T890A, T891A and T892A respectively, but not in the S892P.

rise to a right shift of $V_{1/2}$ significantly after the addition of PMA (Fig. 5). Interestingly, S892P did not change in the voltage dependent activation curve and $V_{1/2}$ value after addition of PMA (after PMA: -39 ± 4.5 mV, before PMA: -35.7 ± 5.7 mV; $n = 6$) (Fig. 5). Furthermore, the effect of PMA on the conductance (at -40 mV) of S892P (64.2 ± 9.6 μ S vs after PMA 57.6 ± 8.8 μ S, $n = 6$) showed no change as compared to wild-type (64.1 ± 3.8 μ S vs after PMA 38.4 ± 2.3 μ S, $n = 8$, $P < 0.05$). In a word, the replacement of serine by proline at residue 892 of CIC-1 causes a loss of the response to the PKC activator PMA.

The properties of S892D

The S892D is designed for the mimicking of phosphorylation at the position 892 and may lead to a constitutive active state of a CIC-1 without the addition of PKC activator. The $V_{1/2}$ value of S892D alone is

Fig. 3. The effects of PMA on S892D mutation. The constitutive phosphorylation of ser892 can be mimicked by mutation of serine to aspartic acid. The wild-type and S892D currents before and after PMA treatment are shown in (A) and (B), respectively. The PMA treatment did not change in the voltage-dependent activation curve of S892D current (D). The superimposed activation curves of S892D, wild-type and wild-type plus PMA (C) are shown in Fig. (E), the $V_{1/2}$ value of S892D alone was close to the wild-type treated with PMA (E).

Fig. 4. Double mutations of T891A/S892A, T891A/T893A and S892A/T893A resulted in the loss of sensitivity to the PMA. Typical current traces and activation curves of double mutants of T891A/S892A, T891A/T893A and S892A/T893A, before and after application of PMA are shown in Fig. (A-C) and Fig. (D-F) respectively. Each of these double mutants treated with PMA did not show the significant changes in $V_{1/2}$ values.

significantly shifted toward more positive potential as compared with the wild-type (Fig. 3; S892D, -10.2 ± 4.9 mV vs. wild-type, -42.9 ± 4.4 mV, $P < 0.05$; $n = 4 - 8$), and can mimic the effect of wild-type treated with PMA (wild-type plus PMA, -13.7 ± 1.7 mV, $n = 8$). There is no significant change in $V_{1/2}$ of S892D between before and after PMA treatment (Fig. 3D and Fig. 5; before PMA: -10.2 ± 4.9 mV, after PMA: -9.6 ± 3.5 mV, $n = 4$). The representative traces of wild-type and S892D in response to PMA stimulation are illustrated in Fig. 3A and 3B, respectively.

Double mutants, T891A/S892A, T891A/T893A and S892A/T893A are less sensitive to PMA

Considering the different effects between the S892P and S892A induced by PMA, the proline may result in a more drastic conformational change than the alanine in a putative PKC-phosphorylation region. This raises the possibility that other sites responsible for the gating effects by PMA might exist in the region around the residue 892.

In order to cause a more drastic change in the putative PKC recognition motif, we designed double mutations of CIC-1. The $V_{1/2}$ values showed no significant differences between double mutants (T891A/S892A, T891A/T893A and S892A/T893A) and wild-type (Fig. 5). However, unlike the wild-type, these three double mutants are resistant to the effect of PMA (Fig. 5). The $V_{1/2}$ values of double mutants T891A/S892A, T891A/T893A and S892A/T893A between before and after PMA treatment are -43.9 ± 3.8 mV vs -36 ± 7.9 mV ($n = 4$), -34.5 ± 4.4 mV vs -32.0 ± 6.1 mV ($n = 4$) and -45.2 ± 4.4 mV vs -38.4 ± 4.5 mV ($n = 5$), respectively. The representative current traces and activation curves, before and after PMA treatment, are shown in the Fig. 4A/4D (for T891A+S892A), Fig. 4B/4E (for T891A+T893A) and Fig. 4C/4F (for S892A+T893A). Figure 5 summarizes the various mutants of CIC-1 that have been analyzed in this study. The $V_{1/2}$ values of wild-type, S682A, S720A, P727L, S740A, T782A, T782R, S798A, S886R, T890A, T891A, S892A, T893A, S896R, S902A, S903A, T929A

and S933A by PMA were significantly shifted to more positive potentials. However, S892P, S892D, T891A/S892A, S892A/T893A and T891A/T893A are no significant changes in $V_{1/2}$ between before and after the treatment with PMA. The conductances (at -40 mV) of these double mutants are also not affected by PMA treatment (Data not shown).

Discussion

In a previous study, Rosenbohm et al. showed that the external application of PMA reduced the human CIC-1 current amplitude expressed in HEK 293 cells [9]. This effect was abolished by perfusion with a specific antagonist of PKC intracellularly and concludes that the human CIC-1 channel activity is regulated by PKC. However, the phosphorylation sites on CIC-1 for PKC have not been determined. The truncation after the CBS1 of CIC-1 C-terminus gave no current [20], which was due to a failure to reach the plasma membrane [20, 21]. It is still not known whether the C-termini of CIC-1 had a role in the regulation of channel activity. The intracellular C-terminal sequences of mammalian CIC proteins are longer than those of the non-mammalian counterparts and are likely to be involved in channel regulation [22]. Human CIC-1 has several putative serine/threonine phosphorylation sites in the C-terminal region. For prediction of phosphorylation sites in CIC-1, NetPhos 2.0 [16] was utilized. NetPhosK 1.0 [17] was also used to find the potential of CIC-1 cytoplasmic domains for their specificity to be phosphorylated by PKC at Ser/Thr. In this study, we constructed a series of mutated CIC-1 genes at the predicted phosphorylation sites and expressed the corresponding proteins in *Xenopus* oocytes. Subsequently, functional analysis of these mutants was performed in order to detect those among them which are not affected by the application of PMA (a PKC activator). The screening for CIC mutations with functional analysis showed that most of the single mutants were a significant right shift in $V_{1/2}$ induced by PMA except the S892P and S892D. Since proline gives the backbone a special rigidity rather than alanine [23], S892P causes a more drastic conformational change of C-terminal domain rather than S892A. This conformational change of S892P may result in loss of response to PMA. In this study we show that the voltage-dependent gating property of S892P (Fig. 2D) is indeed unaffected by PMA (Fig. 2C). This result implied that a sequence containing serine at 892 of CIC-1 could, at least in part, serve as one of the targets for phospho-

Fig. 5. Comparative summary of $V_{1/2}$ of CIC-1 C-terminal mutants by treated with PMA. The $V_{1/2}$ values before and after PMA treatment were indicated as blank and gray bars respectively. Please note that S892P, S892D, T891A/S892A, T891A/T893A and S892A/T893A indicate no significant changes in $V_{1/2}$ between before and after PMA treatment. * $P < 0.05$ as compared with before PMA treatment. N.S.: No significant difference. Measurements are given in mV as mean S.E.M., and $n = 4-8$ in each group from at least two independent batches of oocytes.

rylation by PKC. Although S892A remains the response to the PKC activator, it can be explained that a minor conformational change caused by S892A could not interfere completely with other possible recognition sites for PKC (e.g., Thr890, Thr891 or Thr893).

Substitution of aspartic acid is a generally accepted method to mimic constitutive phosphorylation due to the introduction of a negative-charged functional group [24, 25]. The S892D did result in a significant right shift of $V_{1/2}$ compared with wild-type and to mimic the effect of PMA on wild-type CIC-1 current (Fig. 3B). However, the serine at position of 892 could not be the only phosphorylation site for PKC due to the S892A still have a significant response to PMA. A search through the protein sequence analysis of CIC-1 showed a potential

sequence with multiple phosphorylation sites from Thr890 to Thr893. Therefore, double mutants within the region between Thr891 to Thr893 were generated in this study. Each of these double mutants would expect to make a more drastic change in the protein structure than that of either single mutation alone. All double mutants (T891A/S892A, T891A/T893A and S892A/T893A) were much less sensitive to the effect of PMA (e.g., the $V_{1/2}$ values) than that in each of the single mutants (T891A, S892A and T893A) and the wild-type. However, we cannot rule out the possibility that some mammalian proteins traffic differently when heterologously expressed in *Xenopus* oocytes [26]. The chloride currents properties of each of these double mutants alone (without the drug added) are similar to the wild-type indicated that these mutants may not interfere with their cell membrane surface expression.

PKC typically phosphorylates serine/threonine residues in basic sequences but displays significantly less specificity than protein kinase A [27]. Three amino acid residues in the CIC-1 C-terminus, Thr891, Ser892 and Thr893 (residues from 888 to 896 are RNTTSTRKS), all fitted with the pattern of consensus sequence R/K-X-Ser/Thr or Ser/Thr-X-R/K [27] for PKC. Multiple alignment of human CIC-1 amino acid sequence showed about 87% identity to other mammalian CIC-1 sequences (rat and mouse). A multiple alignment including a sequence

of human CIC-1 Thr891-Ser892-Thr893 is illustrated below.

Human	885-ASFRNTTSTRKSTGA-899
Norway Rat	891-ASFRNTTSTRKTDGG-905
House mouse	891-ASFRNTTSTRKTDGG-905

Human CIC-1 C-terminal residues Thr891 and ser892 are highly conservation, which are correspond to Thr897 and Ser898 of rodent respectively. In this study the results of functional experiments in double mutants, T891A/S892A, S892A/T893A and T891A/T893A, indeed demonstrated that these residues are essential for the effect of PKC activator. Our observations indicate that a region Thr891-Ser892-Thr893 is responsible for the effect of PKC activator on gating properties of CIC-1. These findings provide useful hints for PKC-target sites and add further information on the regulation of CIC-1.

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