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Protein Kinase A Activation Phosphorylates the Rat ClC-2 Cl^- Channel but Does Not Change Activity

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Abstract. Phosphorylation-dependent events have been shown to modulate the activity of several members of the mammalian CLC Cl^- channel gene family, including the inward rectifier ClC-2. In the present study we investigated the regulation of rat ClC-2 expressed in the TSA-201 cell line (a transformed HEK293 cell line that stably expresses the SV40 T-antigen) by protein kinases. Protein kinase A activation phosphorylated ClC-2 in vivo, whereas stimulation of protein kinase C with phorbol 12-myristate 13-acetate did not. In vitro labeling studies confirmed that protein kinase A could directly phosphorylate ClC-2, and that protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II did not. Nevertheless, protein kinase A-dependent phosphorylation of CLC-2 failed to regulate either the magnitude or the kinetics of the hyperpolarization-activated Cl^- currents. Considered together, we demonstrate that protein kinase A activation results in the phosphorylation of rat ClC-2 in vivo, but this event is independent of Cl^- channel activity.

Key words: ClC-2 — Hyperpolarization-activated Cl^- Channel — Phosphorylation — Protein kinase A — Regulation

Introduction

The ubiquitously expressed rat ClC-2 channel, a member of the CLC voltage-activated chloride channel gene family (Jentsch et al., 1995, 1999), exhibits an inward rectifying steady-state current-voltage relation and time-dependent activation at physiological voltages when expressed in *Xenopus* oocytes (Thiemann et al., 1992), DRG neurons (Staley et al., 1996) and HEK293 cells (Park et al., 1998). Similar hyperpolarization-activated Cl^- currents have been associated with the expression of ClC-2 in many different types of cells (Arreola et al., 1996; Carew & Thorn, 1996; Clark et al., 1998; Dinudom, Young & Cook, 1993; Enz, Ross & Cutting, 1999; Ferroni et al., 1997; Huber et al., 1998; Joo et al., 1999; Kajita, Omori & Matsuda, 2000; Murray et al., 1995; Park et al., 1998). Indeed, targeted disruption of the *Clcn2* gene confirmed that hyperpolarization-activated Cl^- currents in Leydig and Sertoli cells are mediated by ClC-2 (Bösl et al., 2001). Other members of the CLC mammalian gene family include ClC-1 that regulates the resting membrane potential in muscle cells (Steinmeyer et al., 1994), the intracellular Cl^- channels ClC-3 (Stobrawa et al., 2001) and ClC-7 (Kornak et al., 2001), isoforms important for renal function—ClC-5, ClC-K (K1 and K2, or Ka and Kb, depending on the species) (Gunther et al., 1998; Kieferle et al., 1994), and isoforms for which the functions are unknown—ClC-4 and ClC-6 (Brandt & Jentsch, 1995).

Regulation of ClC-2 channel activity is multifaceted. The voltage-dependent ClC-2 current is enhanced by low extracellular pH (Jordt & Jentsch, 1997; Sherry et al., 1997; Tewari et al., 2000), is sensitive to the intracellular and extracellular Cl^- concentration (Dinudom et al., 1993; Jordt & Jentsch, 1997), and can be either activated (Furukawa et al., 1998; Thiemann et al., 1992; Xiong et al., 1999) or inhibited (Komwatana et al., 1995) by cell swelling. ClC-2 currents are also apparently modulated by protein kinases. Lipid bilayers fused with membranes from *Xenopus* oocytes injected with rabbit ClC-2 cRNA expressed Cl^- channel activity enhanced by a cAMP-dependent kinase (PKA)-dependent process

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(Sherry et al., 1997). Similarly, the inward-rectifying Cl^- currents in mouse choroid plexus epithelial cells were activated by PKA (Kibble et al., 1997). In contrast, activation of either PKA or protein kinase C (PKC) decreased the amplitude of the ClC-2 inward rectifying currents in human intestinal T84 cells (Fritsch & Edelman, 1996). Human ClC-2 is apparently activated by PKA when expressed in HEK 293 cells (Tewari et al., 2000), whereas, no effect of PKA on this channel was observed in the human bronchial cell line IB3-1 (Schwiebert et al., 1998) or when expressed in *Xenopus* oocytes (Jordt & Jentsch, 1997).

Analysis of the primary amino acid sequence of rat ClC-2 revealed three potential phosphorylation sites for both PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). The three potential PKA phosphorylation sites in rat ClC-2 (Ser82, Ser710 and Thr898) are conserved in mouse, rabbit and human ClC-2 protein. All three sites are predicted to be cytoplasmic on either the amino or carboxy termini of ClC-2, and thus are likely to be accessible to intracellular kinases. Therefore, the modulation of channel activity by kinases may involve direct phosphorylation of the ClC-2 protein, although this has not been previously reported. In the present study, we demonstrate that PKC and CaMKII do not phosphorylate rat ClC-2. In contrast, PKA activation phosphorylated ClC-2 in vivo and in vitro; yet, ClC-2 currents were not modulated by PKA under conditions where the cAMP-dependent Cl^- channel CFTR was activated.

Materials and Methods

Subcloning of ClC-2 and CFTR into Green Fluorescent Protein Expression Vectors

To facilitate identification of transfected cells in patch clamp studies, rat ClC-2 and human CFTR were subcloned into eukaryotic green fluorescent protein (GFP) expression vectors. Cells expressing the channel constructs were identified by epi-fluorescence with an Endow GFP bandpass filter (Chroma Technology, Brattleboro, VT). Rat ClC-2 was subcloned from pCRII-ClC-2 (Park et al., 1998) into the bicistronic expression vector pIRES2-EGFP (Clontech, Palo Alto, CA) using the *Bam*H1 and *Nsi*1 restriction sites for removal from pCRII (Invitrogen, San Diego, CA) and from the *Bam*H1 and *Pst*1 (compatible cohesive end with *Nsi*1) sites for insertion into pIRES-EGFP. A rat ClC-2-egfp fusion construct was made using a PCR-based technique to introduce an *Age*1 restriction enzyme site and to remove the stop codon as described before (Park et al., 1998). Briefly, the open reading frame of rat ClC-2 cDNA was amplified using sense (5′-AAGCAAGAGGAGGCAAGAGGAC-3′) and nonsense primers (5′-GGTCACCGGTAACTTGTCA-3′) with pCRII-ClC-2 as the template, followed by *Age*I digestion. The rat ClC-2 cDNA insert was then subcloned into pEGFP-N1 (Clontech) which had been linearized by *Sma*1 and *Age*1 to construct pClC-2-egfp. The inserts in the bicistronic pClC-2/egfp and fusion pClC-2-egfp constructs were confirmed by sequencing (MJResearch autosequencer).

The open reading frame of human CFTR was amplified from a cDNA in pBluescript-KS(+) provided by Jack R. Riordan (Mayo Clinic Scottsdale, S.C. Johnson Medical Research Center, Scottsdale, AZ). Primers were designed to remove the stop codon and to generate restriction endonuclease sites for subcloning the PCR product into the expression vector pEGFP-N1. The sense primer was 5′-aaactgca-GTAGGTCTTTGGCATTAGGAGCTTG-3′ (lower-case letters indicate a *Pst*1 endonuclease site) and the nonsense primer was 5′-acaccggtAGCCTTGTATCTT-3′ (lower-case letters indicate an *Age*1 endonuclease site). The PCR product containing the open reading frame of CFTR was digested with *Pst*1 and *Age*1 and subcloned into pEGFP-N1 previously digested with *Pst*1 and *Age*1. The pCFTR-egfp construct was sequenced to verify in-frame translation of EGFP fused to the carboxyl terminus of CFTR.

Transient Expression of Rat ClC-2 and Human CFTR-egfp

The TSA-201 cell line (a transformed HEK293 cell line that stably expresses the SV40 T-antigen—kindly provided by Ronald Li and Eduardo Marban, Johns Hopkins University) was used for transient expression of channel proteins. Cells were maintained as previously described (Park et al., 1998) and transiently transfected with ClC-2/egfp bicistronic or CFTR-egfp fusion constructs at 10 μg DNA/100 mm plate using Superfect (Qiagen, Hilden, Germany) and incubated for 48 hours before phosphorylation and electrophysiological studies. In some electrophysiological experiments, HEK293 cells and the ClC-2-egfp fusion construct were used. In this case, cells with positive GFP signals were sorted by their fluorescent intensity using a B-D Vantage cell sorter (Becton Dickinson, Palo Alto, CA). Single cell suspensions at concentration of $1–3 \times 10^6$/ml were prepared in PBS containing 1% FBS and analyzed at a rate of about 1,000 cells/sec using an argon ion laser operating at 488 nm wavelength and 200 milliwatts excitation. Two-parameter gating window (forward scattering vs. fluorescent intensity) was used to define the sorted cell populations. GFP positive cells were collected into a 15-ml conical centrifuge tube filled with 10 ml Dulbecco's modified Eagle's medium containing 20% FBS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml).

In vivo Metabolic $^{32}P_i$ Labeling of Rat ClC-2

To examine whether the ClC-2 is phosphorylated in vivo, TSA-201 cells transiently transfected with the ClC-2/egfp bicistronic construct were incubated for 4 hr in a phosphate-free Dulbecco's modified Eagle's medium (Gibco/BRL, Grand Island, NY) containing 50 μM sodium orthovanadate and 250 μCi/ml $^{32}P_i$. After labeling, the cells were treated for 15 min with either: (1) cAMP cocktail [20 μM forskolin + 100 μM 3-isobutyl-1-methylxanthine + 500 μM 8-(4-chlorophenylthio) cAMP], (2) 0.5 μM Phorbol 12-myristate 13-acetate (PMA), or (3) vehicle. The reaction was stopped by the addition of ice-cold PBS.

Immunoprecipitation and Biochemical Analysis of ClC-2

Following 15 min in vivo treatment with either a cAMP cocktail, PMA or vehicle, ClC-2 transfected TSA-201 cells were washed twice with ice-cold PBS, lysed by incubation at 4°C for 20 min in a modified RIPA buffer [in mM: 50 Tris-HCl (pH 7.4), 500 NaCl, 1 PMSF, and 1 sodium orthovanadate], 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and centrifuged at 10,000 × g for 15 min. ClC-2 protein was immunoprecipitated by incubation of the supernatants at 4°C with

anti ClC-2 antibody (Park et al., 1998) for 4 hr and then treated overnight with protein A-agarose beads (Pierce, Rockford, IL). The immunoprecipitates were resolved by 8.5% SDS/PAGE electrophoresis. After allowing for the decay of ^{32}P, western blot analysis was performed as we described previously (Park et al., 1998) to verify the amount of ClC-2 loaded in each lane.

For in vitro phosphorylation studies of ClC-2, immunoprecipitates from TSA-201 cells transiently transfected with the ClC-2/egfp bicistronic construct were incubated in either a protein kinase A (PKA), protein kinase C (PKC), or calmodulin-dependent protein kinase (CaMKII) assay buffer at 30°C for 60 min in the presence of 10 μCi of [γ-^{32}P]ATP. The reactions were stopped by washing the protein A-agarose beads twice with RIPA buffer followed by two washes with RIPA buffer containing 1 M NaCl. The ClC-2 proteins were recovered by incubation of the beads in 2 × Laemmli SDS sample buffer at 55°C for 15 min and analyzed by 8.5% SDS/PAGE. The fractionated proteins were then electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) and exposed to Kodak Biomax MS film. Western blot analysis was then performed as we described previously (Park et al., 1998). In the PKA reaction, immunoprecipitates were incubated with 1 ng of PKA catalytic subunit (Promega, Madison, WI) in 50 μl reaction buffer containing (in mM) 50 Tris-HCl (pH 7.4), 10 $MgCl_2$, 0.1 ATP, 5 μM cAMP and 100 μg BSA/ml. In the PKC reaction, immunoprecipitates were incubated with 125 ng of PKC (Promega, Madison, WI) in 50 μl reaction buffer containing (in mM) 0.25 EGTA, 0.4 $CaCl_2$, 20 Tris-HCl (pH 7.5), 10 $MgCl_2$, 0.1 ATP, 100 μg BSA/ml, 320 μg phosphatidylserine/ml, and 32 μg diacylglycerol/ml. In the CaMKII reaction, immunoprecipitates were incubated with 100 ng of CaMKII (New England Biolabs, Beverly, MA) in 50 μl reaction buffer containing (in mM) 20 Tris-HCl (pH 7.5), 10 $MgCl_2$, 5 DTT, 0.1 ATP, 0.1 EDTA, 2 $CaCl_2$, and 2.4 μM calmodulin. Kinase activity was normalized to give the same incorporation of phosphate using enzyme-specific peptide substrates for the PKA, PKC, and CaMKII reactions: Kemptide (Promega, Madison, WI), Neurogranin (Promega, Madison, WI), and KRQQSFDLF oligopeptide (New England Biolabs, Beverly, MA), respectively (*data not shown*).

Electrophysiological Analysis

Whole-cell patch clamp recordings were done at room temperature (20–22°C) using an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA). The measured junction potentials for the solutions used were less than 1 mV and so no correction for these was applied. The holding potential was 0 mV. Data acquisition was performed using a 12-bit analog/digital converter controlled by a personal computer.

Currents through expressed Cl^- channels were measured with an external solution in which the impermeant cation *N*-methyl-D-glucamine (NMDG) was used. The composition of this solution was (in mM): 140 NMDG-Cl, 2 $CaCl_2$, 2 $MgCl_2$, 20 HEPES, pH 7.1 (with NMDG). The internal solution used for these recordings contained (mM): 60 Cl, 60 glutamic acid, 120 NMDG, 3 Mg-ATP, 10 EGTA, 10 HEPES, pH of 7.2 (with NMDG). Very shortly after achieving whole-cell mode with these solutions, an outward rectifying current developed in both untransfected and transfected cells. This was likely the volume-sensitive organic osmolyte/anion channel (VSOAC— e.g., (Cannon, Basavappa & Strange, 1998)). This current was transient and disappeared within 10–15 min. The elimination of this current was accelerated by addition of 30 mM mannitol to the external solution. The magnitude and kinetics of expressed ClC-2 and CFTR-egfp were independent of the presence or absence of 30 mM mannitol in the external solution.

Results

In Vivo and In Vitro Phosphorylation of Rat ClC-2 by Protein Kinase A

The primary amino acid sequence of ClC-2 was analyzed for putative phosphorylation sites. Potential cAMP protein kinase phosphorylation sites were identified using a Prosite consensus sequence [RK]2-x-[S/T] (Taylor, 1989), or [RK]2-x-x-[S/T], as described (Feramisco, Glass & Krebs, 1980). As there are a number of exceptions to these rules, an 80% match was used as a threshold value. Rat ClC-2 contains three potential phosphorylation sites for both PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; at Ser82, Ser710 and Thr898), and 10 potential phosphorylation sites for PKC. All PKA sites are conserved in mouse, rabbit and human ClC-2 protein and the localization of these sites to the cytoplasmic amino and carboxy termini of ClC-2 indicates that they are accessible to intracellular kinases.

There is considerable evidence that ClC-2 is regulated by protein kinases (Fritsch & Edelman, 1996; Kibble et al., 1997; Sherry et al., 1997; Tewari et al., 2000); however, the mechanism of regulation has never been directly addressed. To determine whether rat ClC-2 is a substrate for protein kinases in vivo, TSA-201 cells were metabolically labeled with $^{32}P_i$ and then stimulated with either a PKA-activating cocktail or the PKC activator PMA (*see* Methods). Panel *A* of Fig. 1 shows that PKA activation leads to ClC-2 phosphorylation in vivo, but PKC produced little, if any, phosphorylation. To demonstrate the relative amount of ClC-2 protein added to each lane, a western blot of the membrane used in panel *A* was performed using a ClC-2-specific antibody (Fig. 1, panel *B*).

Figure 1 shows that activation of PKA results in phosphorylation of ClC-2 in vivo. However, this experiment does not differentiate between direct phosphorylation of ClC-2 by PKA and phosphorylation of ClC-2 as the consequence of PKA acting through another kinase. To address this issue we immunoprecipitated ClC-2 expressed in TSA-201 cells and then determined whether PKA, PKC, or CaMKII can phosphorylate ClC-2 in vitro. Panel *A* of Fig. 2 shows that in vitro PKA phosphorylated the expressed ClC-2 protein, but PKC and CaMKII failed to do so. To determine the relative amount of ClC-2 loaded in each lane, western blot analysis was performed on the same membrane (panel *B* of Fig. 2). The protein indicated by the solid arrows in panels *A* and *B* is the predicted size for ClC-2 (~100 kDa). The broken arrows point out a much smaller protein (~75 kDa) that is very strongly phosphorylated by PKA (panel *A*) and is labeled by the ClC-2 antibody (panel *B*). The identity of this latter protein is unknown, but likely represents a cleavage product of

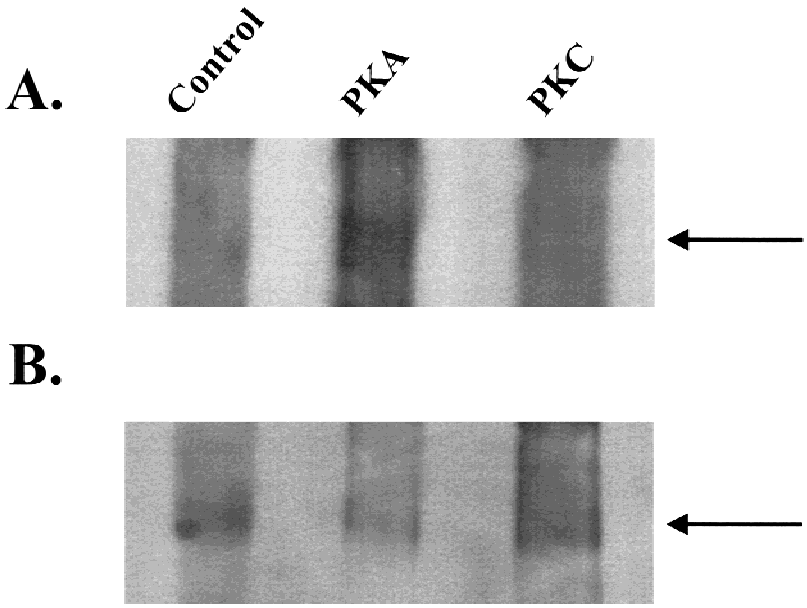

Fig. 1. PKA-dependent phosphorylation of ClC-2 in vivo. Rat ClC-2 was transiently expressed in TSA-201 cells and labeled with $^{32}P_i$. Panel *A*. After labeling, the cells were treated for 15 min with either a PKA-stimulation cocktail [20 μM forskolin + 100 μM 3-isobutyl-1-methylxanthine + 500 μM 8-(4-chlorophenylthio) cAMP], or a PKC activator [0.5 μM Phorbol 12-myristate 13-acetate (PMA)], or vehicle (*Control*). The immunoprecipitates were resolved by 8.5% SDS/PAGE electrophoresis. Panel *B*. To determine the relative amount of ClC-2 loaded in each lane, western blot analysis was performed after allowing for the decay of ^{32}P. The arrows in panels *A* and *B* indicate the position of ClC-2 (~100 kDa).

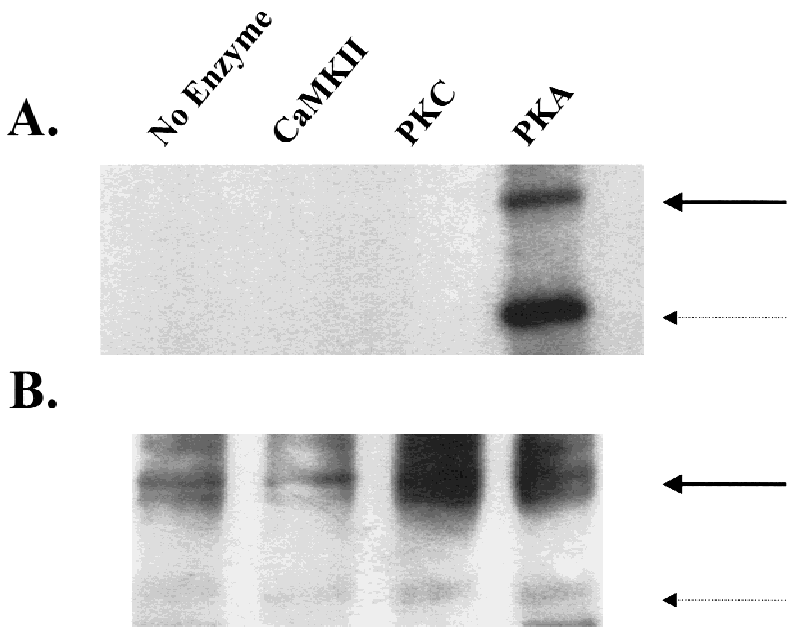

Fig. 2. In vitro labeling of ClC-2 by PKA. Rat ClC-2 was transiently expressed in TSA-201 cells and immunoprecipitated for in vitro labeling studies. Panel *A*. Immunoprecipitated ClC-2 was incubated in the presence of either PKA, PKC, CaMKII or no enzyme and 10 μCi of [γ-^{32}P]ATP, analyzed by 8.5% SDS/PAGE, and transferred to PVDF membrane. Panel *B*. To determine the relative amount of ClC-2 loaded in each lane, western blot analysis was performed after allowing for the decay of ^{32}P. The solid arrows in panels *A* and *B* indicate the position of ClC-2 (~100 kDa). The broken arrow in panels *A* and *B* indicate the position of an ~75 kDa protein.

ClC-2. Moreover, this protein cannot represent the slightly smaller (~97–99 kDa) truncated form of ClC-2 (Cid et al., 2000; Loewen et al., 2000). Together, these experiments indicate that PKA activation phosphorylates ClC-2 and this in vivo event may be direct.

Protein Kinase A Activation Does not Regulate Rat ClC-2 Channel Activity

To determine whether modulation of ClC-2 activity correlates with the phosphorylation induced by increased cAMP, chloride currents were measured using patch-clamp techniques. The ClC-2/egfp bicistronic and ClC-2-egfp fusion constructs were used to facilitate identification of transfected cells expressing ClC-2. Figure 3 shows that treatment with a cAMP cocktail (the same cocktail used for in vivo labeling studies) for 20 min had no significant effect on the magnitude or the activation kinetics (Fig. 3, *inset*) of ClC-2. The average fraction change of the ClC-2 current at −120 mV was 0.99 ± 0.17 after 20 min stimulation ($n = 8$). The properties of the ClC-2 channel-egfp fusion protein ($n = 5$) were comparable to those without the GFP fusion ($n = 3$).

To verify that the stimulation protocol was sufficient to activate channels in TSA-201 cells, a CFTR-egfp fu-

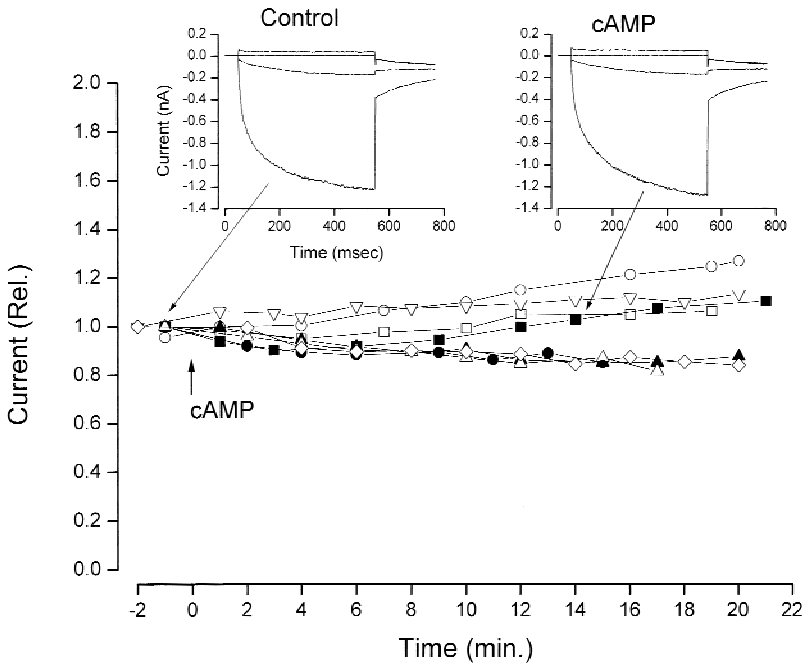

Fig. 3. ClC-2 currents in phosphorylating conditions. Currents at the end of hyperpolarizing pulses to −120 mV (except for △, which was −100 mV) as a function of time of the experiment. Currents normalized to the values recorded 2 min prior to application of the phosphorylating solution. The cAMP phosphorylating solution (*see* Methods) was added at zero time. Each symbol represents a different cell. Currents from cells represented by closed symbols were recorded with a solution of normal tonicity; 30 mM mannitol was added for the cells indicated by open symbols. Experiments represented by △, ▽, and ◇ were done without the GFP fusion, whereas those represented by ▲, □, ■, ○, and ● were done with the ClC-2-egfp fusion. Insets: currents recorded at −120, −60, and +60 mV from the 0 mV holding potential. Tails recorded at −50 mV following the test pulse. Currents recorded from the indicated cell at the indicated times.

sion construct was used to express human CFTR. Figure 4 shows that the maximal response to the cAMP cocktail in most cells occurred in less than 5 min—the slowest responding cell showed effects within 5 min but the maximal response wasn't until 10 min. The average current at −80 mV before cAMP addition was −0.029 ± 0.006 nA in CFTR expressing cells. In contrast, the average current after cAMP addition was −6.1 ± 0.85 nA (SEM, $n = 6$), a greater than 200-fold increase in the current amplitude.

Discussion

ClC-2 is a voltage activated Cl^- channel that has wide tissue distribution suggesting that it may play an important functional housekeeping role in many different cell types. In the stomach, ClC-2 appears to be activated by low external pH, and thus could be involved in HCl secretion (Malinowska et al., 1995; Sherry et al., 1997; Stroffekova et al., 1998). ClC-2 is highly expressed in the brain (Thiemann et al., 1992), where it has been hypothesized to regulate the intracellular Cl^- concentration of neurons (Staley et al., 1996). According to this model, ClC-2 expression would produce a large negative shift in the Cl^- equilibrium potential and influence the production of action potentials. In the lung, ClC-2 expression is developmentally regulated, expression levels decreasing dramatically shortly after birth (Blaisdell et al., 1999; Chu et al., 1999; Murray, Chu & Zeitlin, 1996; Murray et al., 1995). Its targeting to the apical membrane of lung cells suggests that it may play an important role in Cl^- and water secretion necessary for the development of this organ (Blaisdell et al., 2000). However, ClC-2-deficient mice were normal for these proposed functions; targeted disruption of the *Clcn2* gene leads to severe degeneration of male germ cells and photoreceptors (Bösl et al., 2001).

Understanding the acute regulation of this protein may have important clinical implications. In the present study, we show that rat ClC-2 is phosphorylated following PKA activation in vivo, and is directly phosphorylated by PKA in vitro. Nevertheless, the currents generated by these channels are not affected by this PKA-induced modification. In contrast, PKC (neither in vivo nor in vitro) and CaMKII (in vitro) failed to phosphorylate rat ClC-2. However, we cannot rule out the possibility that PKC and/or CaMKII activation might indirectly regulate ClC-2 channel activity. Our results are consistent with those of Schwiebert et al. (1998) and Jordt and Jentsch (1997) where no effect of PKA on the human ClC-2 channel was observed. Conversely, activation of PKA decreased the amplitude of the ClC-2 inward rectifying currents in human intestinal T84 cells (Fritsch & Edelman, 1996). However, ClC-2-like currents in mouse choroid plexus epithelial cells (Kibble et al., 1997) and human ClC-2 currents expressed in HEK293 cells (Tewari et al., 2000) are apparently activated by PKA. The simplest explanations for these differences are that PKA-dependent regulation of ClC-2 is cell-type or species-specific. In Fig. 3 we show the rat ClC-2 currents expressed in HEK293 and TSA-102 cells. In both cases, PKA activation failed to have an effect on ClC-2 current amplitude or activation kinetics. Thus, the cell type used for expression of ClC-2 cannot explain the difference in this case. Moreover, currents produced by

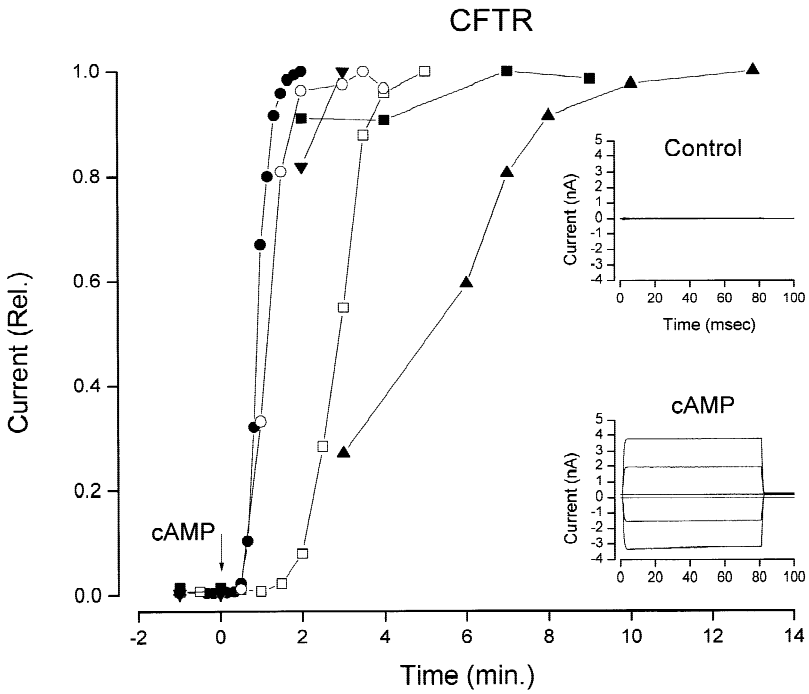

Fig. 4. CFTR currents in phosphorylating conditions. Currents at the end of hyperpolarizing pulses to −80 mV as a function of time of the experiment. Currents normalized by the largest recorded value. The cAMP phosphorylating solution (*see* Methods) was added at zero time. Each symbol represents a different cell. Insets: currents recorded at −60, −30, +30, and +60 mV from the 0 mV holding potential. Currents recorded from the cell identified by ▲ before and after 10 min in the phosphorylating solution.

human ClC-2 when expressed in HEK293 cells (Tewari et al., 2000) show properties quite different from those seen for human ClC-2 when expressed in other cell types or for ClC-2 from other species. Specifically, both "trademarks" of ClC-2, the inward rectification and the time-dependent activation, are absent (Tewari et al., 2000).

In conclusion, we show protein kinase A activation phosphorylates rat ClC-2 in vivo, but this activation is independent of Cl^- channel activity. This has important implications as to the mechanism by which ClC-2 activity is acutely regulated.

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References

Arreola, J., Park, K., Melvin, J.E., Begenisich, T. 1996. Three distinct chloride channels control anion movements in rat parotid acinar cells. *J. Physiol.* **490:**351–362

Blaisdell, C.J., Edmonds, R.D., Wang, X.T., Guggino, S., Zeitlin, P.L. 2000. pH-regulated chloride secretion in fetal lung epithelia. *Am. J. Physiol.* **278:**L1248–L1255

Blaisdell, C.J., Pellettieri, J.P., Loughlin, C.E., Chu, S., Zeitlin, P.L. 1999. Keratinocyte growth factor stimulates ClC-2 expression in primary fetal rat distal lung epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* **20:**842–847

Bösl, M.R., Stein, V., Hübner, C., Zdebik, A.A., Jordt, S.-E., Mukhopadhyay, A.K., Davidoff, M.S., Holstein, A.-F., Jentsch, T.J. 2001. Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon ClC-2 Cl^- channel disruption. *EMBO J.* **20:**1289–1299

Brandt, S., Jentsch, T.J. 1995. ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett.* **377:**15–20

Cannon, C.L., Basavappa, S., Strange, K. 1998. Intracellular ionic strength regulates the volume sensitivity of a swelling-activated anion channel. *Am. J. Physiol.* **275:**C416–C422

Carew, M.A., Thorn, P. 1996. Identification of ClC-2-like chloride currents in pig pancreatic acinar cells. *Pfluegers Arch.* **433:**84–90

Chu, S., Blaisdell, C.J., Liu, M.Z., Zeitlin, P.L. 1999. Perinatal regulation of the ClC-2 chloride channel in lung is mediated by Sp1 and Sp3. *Am. J. Physiol.* **276:**L614–L624

Cid, L.P., Niemeyer, M.I., Ramirez, A., Sepulveda, F.V. Splice variants of a ClC-2 chloride channel with differing functional characteristics. *Am. J. Physiol.* **279:**C1198–C1210

Clark, S., Jordt, S.E., Jentsch, T.J., Mathie, A. 1998. Characterization of the hyperpolarization-activated chloride current in dissociated rat sympathetic neurons. *J. Physiol.* **506:**665–678

Dinudom, A., Young, J.A., Cook, D.I. 1993. Na^+ and Cl^- conductances are controlled by cytosolic Cl^- concentration in the intralobular duct cells of mouse mandibular glands. *J. Membrane Biol.* **135:**289–295

Enz, R., Ross, B.J., Cutting, G.R. 1999. Expression of the voltage-gated chloride channel ClC-2 in rod bipolar cells of the rat retina. *J. Neurosci.* **19:**9841–9847

Feramisco, J.R., Glass, D.B., Krebs, E.G. 1980. Optimal spatial requirements for the location of basic residues in peptide substrates for the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **255:**4240–4245

Ferroni, S., Marchini, C., Nobile, M., Rapisarda, C. 1997. Characterization of an inwardly rectifying chloride conductance expressed by cultured rat cortical astrocytes. *Glia* **21:**217–227

Fritsch, J., Edelman, A. 1996. Modulation of the hyperpolarization-

activated Cl^- current in human intestinal T84 epithelial cells by phosphorylation. *J. Physiol.* **490:**115–128

Furukawa, T., Ogura, T., Katayama, Y., Hiraoka, M. 1998. Characteristics of rabbit ClC-2 current expressed in *Xenopus* oocytes and its contribution to volume regulation. *Am. J. Physiol.* **274:**C500–C512

Gunther, W., Luchow, A., Cluzeaud, F., Vandewalle, A., Jentsch, T.J. 1998. ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl. Acad. Sci. USA* **95:**8075–8080

Huber, S., Schroppel, B., Kretzler, M., Schlondorff, D., Horster, M. 1998. Single cell RT-PCR analysis of ClC-2 mRNA expression in ureteric bud tip. *Am. J. Physiol.* **274:**F951–F957

Jentsch, T.J., Friedrich, T., Schriever, A., Yamada, H. 1999. The CLC chloride channel family. *Pfluegers Arch.* **437:**783–795

Jentsch, T.J., Gunther, W., Pusch, M., Schwappach, B. 1995. Properties of voltage-gated chloride channels of the ClC gene family. *J. Physiol.* **482:**19S–25S

Joo, N.S., Clarke, L.L., Han, B.H., Forte, L.R., Kim, H.D. 1999. Cloning of ClC-2 chloride channel from murine duodenum and its presence in CFTR knockout mice. *Biochim. Biophys. Acta* **1446:**431–437

Jordt, S.E., Jentsch, T.J. 1997. Molecular dissection of gating in the ClC-2 chloride channel. *Embo J.* **16:**1582–1592

Kajita, H., Omori, K., Matsuda, H. 2000. The chloride channel ClC-2 contributes to the inwardly rectifying Cl^- conductance in cultured porcine choroid plexus epithelial cells. *J. Physiol.* **523:**313–324

Kieferle, S., Fong, P., Bens, M., Vandewalle, A., Jentsch, T.J. 1994. Two highly homologous members of the ClC chloride channel family in both rat and human kidney. *Proc. Natl. Acad. Sci. USA* **91:**6943–6947

Kibble, J.D., Garner, C., Colledge, W.H., Brown, S., Kajita, H., Evans, M., Brown, P.D. 1997. Whole cell Cl^- conductances in mouse choroid plexus epithelial cells do not require CFTR expression. *Am. J. Physiol.* **272:**C1899–1907

Komwatana, P., Dinudom, A., Young, J.A., Cook, D.I. 1995. Osmotic sensitivity of the hyperpolarization-activated Cl^- current in mouse mandibular duct cells. *Cell Physiol. Biochem.* **5:**243–251

Kornak, U., Kasper, D., Bösl, M.R., Kaiser, E., Schweizer, M., Schulz, A., Friedrich, W., Delling, G., Jentsch, T.J. 2001. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* **104:**205–215

Loewen, M.E., MacDonald, D.W., Gaspar, K.J., Forsyth, G.W. Isoform-specific exon skipping in a variant form of ClC-2. *Biochim. Biophys. Acta* **1493:**284–288

Malinowska, D.H., Kupert, E.Y., Bahinski, A., Sherry, A.M., Cuppoletti, J. 1995. Cloning, functional expression, and characterization of a PKA-activated gastric Cl^- channel. *Am. J. Physiol.* **268:**C191–200

Murray, C.B., Chu, S., Zeitlin, P.L. 1996. Gestational and tissue-specific regulation of C1C-2 chloride channel expression. *Am. J. Physiol.* **271:**L829–L837

Murray, C.B., Morales, M.M., Flotte, T.R., McGrath-Morrow, S.A., Guggino, W.B., Zeitlin, P.L. 1995. CIC-2: a developmentally dependent chloride channel expressed in the fetal lung and downregulated after birth. *Am. J. Respir. Cell. Mol. Biol.* **12:**597–604

Park, K., Arreola, J., Begenisich, T., Melvin, J.E. 1998. Comparison of voltage-activated Cl^- channels in rat parotid acinar cells with ClC-2 in a mammalian expression system. *J. Membrane Biol.* **163:**87–95

Schwiebert, E.M., Cid-Soto, L.P., Stafford, D., Carter, M., Blaisdell, C.J., Zeitlin, P.L., Guggino, W.B., Cutting, G.R. 1998. Analysis of ClC-2 channels as an alternative pathway for chloride conduction in cystic fibrosis airway cells. *Proc. Natl. Acad. Sci. USA* **95:**3879–3884

Sherry, A.M., Stroffekova, K., Knapp, L.M., Kupert, E.Y., Cuppoletti, J., Malinowska, D.H. 1997. Characterization of the human pH- and PKA-activated ClC-2G(2 alpha) Cl^- channel. *Am. J. Physiol.* **273:**C384–C393

Staley, K., Smith, R., Schaack, J., Wilcox, C., Jentsch, T.J. 1996. Alteration of GABAA receptor function following gene transfer of the CLC-2 chloride channel. *Neuron* **17:**543–551

Steinmeyer, K., Lorenz, C., Pusch, M., Koch, M.C., Jentsch, T.J. 1994. Multimeric structure of ClC-1 chloride channel revealed by mutations in dominant myotonia congenita (Thomsen). *Embo J.* **13:**737–743

Stobrawa, S.M., Breiderhoff, T., Takamori, S., Engel, D., Schweizer, M., Zdebik, A.A., Bosl, M.R., Ruether, K., Jahn, H., Draguhn, A., Jahn, R., Jentsch, T.J. 2001. Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* **29:**185–196

Stroffekova, K., Kupert, E.Y., Malinowska, D.H., Cuppoletti, J. 1998. Identification of the pH sensor and activation by chemical modification of the ClC-2G Cl^- channel. *Am. J. Physiol.* **275:**C1113–C1123

Taylor, S.S. 1989. cAMP-dependent protein kinase. Model for an enzyme family. *J. Biol. Chem.* **264:**8443–8446

Tewari, K.P., Malinowska, D.H., Sherry, A.M., Cuppoletti, J. 2000. PKA and arachidonic acid activation of human recombinant ClC-2 chloride channels. *Am. J. Physiol.* **279:**C40–C50

Thiemann, A., Grunder, S., Pusch, M., Jentsch, T.J. 1992. A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* **356:**57–60

Xiong, H., Li, C., Garami, E., Wang, Y., Ramjeesingh, M., Galley, K., Bear, C.E. 1999. ClC-2 activation modulates regulatory volume decrease. *J. Membrane Biol.* **167:**215–221