Sites of Protein Kinase A Activation of the Human ClC-2 Cl\textsuperscript{−} Channel*

Received for publication, November 17, 2003, and in revised form, March 4, 2004 Published, JBC Papers in Press, March 9, 2004, DOI 10.1074/jbc.M312567200

John Cuppoletti, Kirti P. Tewari, Ann M. Sherry, Christopher J. Ferrante, and Danuta H. Malinowska

From the Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0576

Human ClC-2 Cl\textsuperscript{−} (hClC-2) channels are activated by protein kinase A (PKA) and low extracellular pH\textsubscript{o}. Both of these effects are prevented by the PKA inhibitor, myristoylated PKI. The aims of the present study were to identify the PKA phosphorylation site(s) important for PKA activation of hClC-2 at neutral and low pH\textsubscript{o} and to examine the relationship between PKA and low pH\textsubscript{o} activation. Recombinant hClC-2 with point mutations of consensus phosphorylation sites was prepared and stably expressed in HEK-293 cells. The responses to forskolin plus isobutylmethylxanthine at neutral and acidic pH\textsubscript{o} were studied by whole cell patch clamp in the presence and absence of phosphatase inhibitors. The double phosphorylation site (RRAT655(A) plus RGET691(A)) mutant hClC-2 lost PKA activation and low pH\textsubscript{o} activation. Either RRAT or RGET was sufficient for PKA activation of hClC-2 at pH\textsubscript{o} 7.4, as long as phosphatase inhibitors (cyclosporin A or endothal) were present. At pH\textsubscript{o} 6 only RGET was needed for PKA activation of hClC-2. Low pH\textsubscript{o} activation of hClC-2 Cl\textsuperscript{−} channel activity was PKA-dependent, retained in RGET(A) mutant hClC-2, but lost in RRAT(A) mutant hClC-2. RRAT655(D) mutant hClC-2 was constitutively active and was further activated by PKA at pH\textsubscript{o} 7.4 and 6.0, consistent with the above findings. These results show that activation of hClC-2 is differentially regulated by PKA at two sites, RRAT655 and RGET691. Either RRAT655 or RGET91 was sufficient for activation at pH\textsubscript{o} 7.4. RGET, but not RRAT, was sufficient for activation at pH\textsubscript{o} 6.0. However, in the RGET91(D) mutant, there was PKA activation at pH\textsubscript{o} 6.0.

Human ClC-2 Cl\textsuperscript{−} (hClC-2) channels are activated by PKA, an activation that is prevented by treatment with a permeant PKA inhibitor, myristoylated protein kinase inhibitor, mPKI, to determine the structural basis for PKA activation and PKA-dependent low pH\textsubscript{o} activation of hClC-2.

* This work was supported by National Institutes of Health NIDDK Grant DK43816 and NHLBI Grant HL58399 (to J. C. and D. H. M.) and United States Department of Defense Grant ARO MURI DAAD 19-02-1-0227 (to J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 513-558-3022; Fax: 513-558-5798; E-mail: John.Cuppoletti@uc.edu.

‡ The abbreviations used are: hClC-2, human ClC-2 Cl\textsuperscript{−} channel; HEK-293, human epithelial kidney cell line; IBMX, isobutylmethylxanthine; mPKI, protein kinase inhibitor 14-22 amide myristoylated Myr\textsuperscript{−}Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH\textsubscript{2}, trifluoroacetate salt; PKA, cyclic adenosine monophosphate-dependent protein kinase; PP, protein phosphatase.
EXPERIMENTAL PROCEDURES

Transfected HEK-293 Cells—HEK-293 cells stably transfected with wild-type or mutant human CIC-2 cDNA in the mammalian expression vector pcDNA3.1 using LipofectAMINE were prepared as described previously (2).

Site-directed Mutagenesis—Site-directed mutagenesis of single phosphorylation sites was carried out using a Transformer site-directed mutagenesis kit (Clontech). Four mutants, as follows, were prepared and confirmed by sequencing: RRAT655(acc); RRQS655, RGET691(acc)→Ag(cec); a double mutant containing both of these changes; and RRAT655(acc)→Dgac). The entire cDNA was also verified by sequencing. A PCR fragment was used to introduce the point mutation into plasmid vector pPC2.1Topo. After confirming the sequence, a SandHI/HindIII fragment, which included the mutation, was cloned into the full-length human CIC-2 cDNA and sequenced over the ligation sites.

Measurement of Whole Cell Cl− Currents—Whole cell currents were measured as described previously (5). Forskolin and arachidonic acid in MeSO were diluted into the bath solution resulting in a final concentration of MeSO of 0.1% or less. Controls containing equivalent amounts of MeSO alone were always performed. Statistical significance of the difference between two means was determined using the Student's t test using N = number of cells.

Materials—HEK-293 cells were obtained from American Type Culture Collection (ATCC). HEPES, MeSO, Tris, EGTA, and inorganic and organic salts were obtained from Sigma. Forskolin, arachidonic acid, cyclosporin A, nodularin, endothal, and mPKI were from Calbiochem. IBMX was from Aldrich. LipofectAMINE, pcDNA3.1, and RCVS76 were from Invitrogen.

RESULTS

Wild-type and Mutant hCIC-2 Cl− Channel Activity at pHo 7.4—Fig. 1A shows typical Cl− current recordings from HEK-293 cells expressing wild-type CIC-2 Cl− channels at pH7.4 with no addition (control) and following addition of 5 μM forskolin plus 20 μM IBMX (forskolin/IBMX). The cells were subsequently treated with 1 μM arachidonic acid. Both treatments increased Cl− currents. Similar treatments of nontransfected HEK-293 cells had no effects (Table IV). Cl− currents in HEK-293 cells expressing the double site (RGET(A) plus RRAT(A)) or the single site (RRAT(A)) mutant cIC-2 Cl− channel were not activated by forskolin/IBMX but were activated by arachidonic acid. This suggests that RRAT is involved in the PKA activation of hCIC-2 at pH7.4. HEK-293 cells expressing the single site RGET(A) mutant CIC-2 were activated by forskolin/IBMX, and the level of activation was higher than in HEK-293 cells expressing wild-type CIC-2, indicating that the remaining site, RRAT, is involved in PKA activation of the channel at pH7.4. The single site RRAT(D) mutant hCIC-2 showed high basal Cl− channel activity that was further increased with forskolin/IBMX. The RGET(D) site was also constitutively active, thus confirming the importance of these sites to PKA activation of hCIC-2 at pH7.4. IV curves normalized to capacitance under each condition are shown in Fig. 1B. The summarized data expressed as slope conductance (nS/pF) are shown in Fig. 1C and Table II. The extent of arachidonic acid activation was similar in wild-type and mutant channels, demonstrating that the mutant hCIC-2 channels were expressed to levels similar to that of the wild-type channel in HEK-293 cells.

Wild-type and Mutant hCIC-2 Cl− Channel Activity at pHo 6.0—Fig. 2A shows typical Cl− current recordings of wild-type and mutant hCIC-2 channels stably expressed in HEK-293 cells, first recorded at pH7.4 and then recorded at pH6.0, followed by forskolin/IBMX and then arachidonic acid. The IV curves normalized to capacitance are shown in Fig. 2B, and the summarized data expressed as slope conductance (nS/pF) are shown in Fig. 2C and Table III. Nontransfected HEK-293 cells had very low currents, and these treatments had no effect on the current (Table IV). Wild-type and RGET(A) mutant hCIC-2 channels were activated by reduction of pHo, whereas low pHo activation was lost in the RRAT(A) plus RGET(A), RRAT(A), RRAT(D), and RGET(D) mutant hCIC-2 (Fig. 2 and Table III). This result demonstrated that RRAT was involved in low pHo activation, presumably as a result of basal level phosphorylation. The remaining RGET site did not support low pHo activation, suggesting separate roles for RRAT and RGET in low pHo activation and in PKA-dependent activation at reduced pHo levels. At pH6, the wild-type channel and the RRAT(A), RRAT(D), and RGET(D) mutant channels were activated by forskolin/IBMX, but the RRAT(A) plus RGET(A) and the RGET(A) mutant channels did not respond to forskolin/IBMX. These findings, coupled with the loss of low pHo activation of the RRAT(D) mutant, are consistent with RGET involvement in forskolin/IBMX activation at pH6 and with a separate role for RRAT in low pHo activation. The RGET(D) mutant showed PKA activation at pH6.0. This showed that RRAT supports PKA-dependent activation at pH6.0, although RRAT alone did not (Table III).

Role of Basal State Phosphorylation in Low pHo Activation—Fig. 3 and Table V show that low pHo activation of wild-type hCIC-2 and RGET(A) mutant hCIC-2 was abolished by mPKI. Thus, acid activation in the basal state is dependent upon PKA phosphorylation of RRET.

Effect of Protein Phosphatase Inhibitors—To assess whether protein phosphatase inhibition could affect basal levels or forskolin/IBMX-activated levels of wild-type hCIC-2 Cl− channel activity, the effects of cyclosporin A (a PP2B inhibitor) (9) and endothenal (a PP2A >> PP1 inhibitor) (10) were examined at pH7.4 and 6.0. As shown in Table II, at pH7.4 cyclosporin A (20 μM) added to the bath significantly increased (approximately doubled) the basal Cl− channel slope conductance of wild-type and both single-site mutant hCIC-2 channels, but it was without effect on the double phosphorylation site mutant hCIC-2. Entaking (500 nm) had no effect on basal activity of either wild-type or mutant channels. However both cyclosporin A and endothenal increased forskolin/IBMX activation of the wild-type channel and rescued the forskolin/IBMX activation of the RRAT(A) mutant hCIC-2 at pH7.4 (Fig. 4, Table II). These results show that both RRAT and RGET phosphorylation play a role in activation of hCIC-2 at pH7.4. Suppression of phosphatase activity at pH7.4 was required to show activation of the RRAT(A) mutant channel, which retains the phosphatase-sensitive RGET site. When 1 μM nodularin, a cell-impermeant phosphatase inhibitor with a specificity of PP1(≥)PP2A >> PP2B, was added to the bath, there was no effect; however, when it was added to the pipette solution the wild-type channel showed increased activation by forskolin/IBMX, but the basal level was unaffected at pH7.4 (Table II). In contrast, at pH6 the only significant phosphatase inhibitor effects were those of cyclosporin A and nodularin (in the pipette), which increased forskolin/IBMX activation of the wild-type hCIC-2 channel. No

---

**Table I**

| Human | Rabbit | Rat | Location |
|-------|-------|-----|----------|
| RRAT  | RRQS  | C terminus (A) | | 651 |
| RGET  | RGETS | C terminus (A) | | 691 |
| KRKP  | KRKPS | C terminus (A) | | 702 |
| KRKS  | KRKS  | C terminus (B) | | 69 |
| RIS   | RIS   | C terminus (B) | | 55 |
| RTS   | RTS   | C terminus (B) | | 52 |
| RDS   | RDS   | C terminus (B) | | 60 |
| REGS  | REGS  | C terminus (B) | | 60 |
| RTY   | RTY   | N terminus (B) | | 27 |
| RVCS  | RVCS  | N terminus (B) | | 76 |

A = PKA, protein kinase C (PKC), CaMKII sites; B = PKA, PKC sites; novel sites are bolded.
Fig. 1. Effect of PKA activation on wild-type (WT) and phosphorylation site mutant hClC-2 Cl⁻ channel activity at pH 7.4. A, representative scans of Cl⁻ currents of HEK-293 cells expressing wild-type and RRAT(A) plus RGET(A), RRAT(A), RGET(A), and RRAT(D) mutant hClC-2 Cl⁻ channels before (C, control) and after treatment with 5 μM forskolin, 20 μM IBMX (F/I) followed by 1 μM arachidonic acid (AA). B, IV curves for Cl⁻ currents normalized to cell capacitance (pA/pF). C, summarized data expressed as normalized slope conductance (nS/pF) for the data given in A and B. Data in B and C were plotted as means ± S.E. Numbers in parentheses, number of cells and detailed statistical comparisons for each condition are shown in Table II. #, p < 0.01, and *, p < 0.001, with respect to control.
|                | Wild Type | RRAT(A) | RGET(A) | Double Mutant | RRAT(D) | RGET(D) | WT vs RRAT(A) | WT vs RGET(A) | WT vs Double | WT vs (i) RRAT(D) | WT vs (ii) RGET(D) |
|----------------|-----------|---------|---------|---------------|---------|---------|--------------|--------------|--------------|----------------|-----------------|
| Control pH 7.4| 0.043 ± 0.005 (14) | 0.071 ± 0.006 (22) | 0.042 ± 0.005 (18) | 0.208 ± 0.005 (3) | 0.367 ± 0.013 (12) | 0.286±0.022 (15) | NS | NS | P<0.001 | (i) P<0.001 | (ii) P<0.001 |
| F/I            | 0.170 ± 0.011 (9) | 0.081 ± 0.017 (6) | 0.470 ± 0.014 (5) | 0.135 ± 0.006 (3) | 0.464 ± 0.026 (4) | 0.239±0.010 (3) | NS vs control | P<0.01 | NS | (i) P<0.001 | (ii) P<0.01 |
| F/I + AA       | 0.437 ± 0.032 (5) | 0.500 ± 0.081 (6) | 0.529 ± 0.016 (5) | 0.458 ± 0.013 (3) | 0.516 ± 0.024(4) | 0.346±0.013 (3) | P<0.05 vs control | NS vs F/I | NS | (i) NS | (ii) P<0.05 |
| AA alone       | 0.437 ± 0.021 (5) | 0.558 ± 0.027 (5) | 0.500 ± 0.021 (5) | 0.619 ± 0.013(4) | 0.0<0.01 vs control | NS | NS | P<0.001 | (i) P<0.001 | NS | (ii) NS |
| Cyclo          | 0.072 ± 0.009 (10) | 0.123 ± 0.001 (5) | 0.099 ± 0.012 (7) | 0.178 ± 0.004 (3) | 0.346±0.011 (3) | P<0.05 vs control | NS | NS | P<0.001 | (i) P<0.001 | NS | (ii) NS |
| Cyclo + F/I    | 0.371 ± 0.066 (5) | 0.310 ± 0.066 (5) | 0.390 ± 0.009 (3) | 0.103 ± 0.010 (3) | 0.30±0.030 (3) | NS vs control | NS | NS | P<0.02 | (i) NS | (ii) NS |
| Cyclo + F/I + AA| 0.547 ± 0.089 (5) | 0.420 ± 0.051 (3) | 0.550 ± 0.026 (3) | 0.30±0.020 (3) | 0.475±0.021 (3) | P<0.02 vs cyclo/F + I | NS | NS | NS | (i) NS | (ii) NS |
| Endo           | 0.064 ± 0.008 (3) | 0.071 ± 0.008 (7) | 0.072 ± 0.008 (6) | NS vs control | NS | NS | NS | NS | P<0.01 | NS | NS |
| Endo + F/I     | 0.320 ± 0.019 (3) | 0.588 ± 0.038 (3) | 0.367 ± 0.018 (3) | 0.367±0.018 (3) | 0.0<0.01 vs control | NS vs control | NS | NS | NS | NS | NS |
| Nod-bath       | 0.058 ± 0.001 (3) | 0.051 ± 0.001 (3) | 0.051 ± 0.001 (3) | 0.0<0.01 vs control | NS | NS | NS | NS | NS | NS | NS |
| Nod-bath + F/I | 0.170 ± 0.012 (3) | 0.081 ± 0.017 (6) | 0.135 ± 0.006 (3) | 0.464 ± 0.026 (4) | 0.239±0.010 (3) | NS vs control | P<0.01 | NS | (i) P<0.001 | (ii) P<0.01 |
| Nod-bath + F/I + AA | 0.346 ± 0.038 (3) | 0.500 ± 0.081 (6) | 0.529 ± 0.016 (5) | 0.458 ± 0.013 (3) | 0.516 ± 0.024(4) | 0.346±0.013 (3) | P<0.05 vs control | NS vs F/I | NS | (i) NS | (ii) P<0.05 |
| Nod-pip        | 0.102 ± 0.033 (4) | 0.558 ± 0.027 (5) | 0.500 ± 0.021 (5) | 0.619 ± 0.013(4) | 0.0<0.01 vs control | NS | NS | P<0.001 | (i) P<0.001 | NS | (ii) NS |
| Nod-pip + F/I  | 1.032 ± 0.201 (4) | 0.558 ± 0.027 (5) | 0.500 ± 0.021 (5) | 0.619 ± 0.013(4) | 0.0<0.01 vs control | NS | NS | P<0.001 | (i) P<0.001 | NS | (ii) NS |

Effects of forskolin/IBMX, arachidonic acid, and phosphatase inhibitors are shown. NS, not significant. Forskolin/IBMX (F/I), 5 μM/20 μM; arachidonic acid (AA), 1 μM; cyclosporin A (cyclo), 20 μM; endothal (endo), 500 nM; nodularin (nod), 1 μM; values in brackets show significant decreases. pip, pipette solution.
Fig. 2. Effect of PKA activation on wild-type (WT) and phosphorylation site mutant hClC-2 Cl⁻ channel activity at pH 6. A, representative scans of Cl⁻ currents of HEK-293 cells expressing human wild-type and RRAT(A), RRAT(A), RGET(A), and RRAT(D) mutant hClC-2 Cl⁻ channels before (C, control) and after treatment with 5 μM forskolin, 20 μM IBMX (F/I) followed by 1 μM arachidonic acid (AA). B, I/V curves for Cl⁻ currents normalized to cell capacitance (pA/pF). C, summarized data expressed as normalized slope conductance (nS/pF) for the data in A and B. Data in B and C were plotted as means ± S.E. Numbers in parentheses, number of cells and detailed statistical comparisons for each condition are shown Table III. ##, p < 0.05; #, p < 0.01; *, p < 0.001, with respect to control.
| pH 6 vs 7.4 | Wild Type | RRAT(A) | RGET(A) | Double Mutant | RRAT(D) | RGET(D) | WT vs RRAT(A) | WT vs RGET(A) | WT vs Double | WT vs (i) RRAT(D) (ii) RGET(D) |
|------------|-----------|---------|---------|---------------|---------|---------|---------------|---------------|--------------|-------------------------------|
| Control   | pH 7.4    | 0.044 ± 0.004(3) | 0.053 ± 0.013(5) | 0.088 ± 0.005 (5) | 0.190 ± 0.014(3) | 0.367 ± 0.013(12) | 0.286 ± 0.022(15) | NS | P<0.001 | P<0.01 |
| pH 6       | NS vs cont | 0.153 ± 0.021(3) | P<0.05 vs cont | 0.047 ± 0.022(5) | 0.173 ± 0.023(5) | 0.175 ± 0.016(3) | 0.381 ± 0.025(3) | NS vs control | 0.283 ± 0.029 (9) | NS vs control |
| pH 6 + F/I | P<0.01 vs pH 6 | 0.276 ± 0.015(3) | P<0.01 vs pH 6 | 0.432 ± 0.054(5) | 0.171 ± 0.024(5) | 0.168 ± 0.033(3) | 0.511 ± 0.035 (3) | NS vs pH 6 | 0.707 ± 0.046 (4) | P<0.001 vs pH 6 |
| pH 6 + AA  | P<0.01 vs cont | 0.598 ± 0.018(3) | P<0.01 vs pH 6 | 0.303 ± 0.069(5) | 0.303 ± 0.031(5) | 0.476 ± 0.020(3) | 0.774 ± 0.064 (4) | NS vs pH 6 + F/I | 0.005 vs cont | NS vs pH 6 + F/I |
| pH 6 + cyclo + F/I | 0.158 ± 0.0027(5) | NS vs pH 6 | 0.087 ± 0.008(3) | 0.185 ± 0.030(3) | 0.185 ± 0.030(3) | 0.313 ± 0.028 (5) | NS | NS | NS | P<0.01 |
| pH 6 + cyclo + F/I + AA | 0.563 ± 0.062(5) | NS vs pH 6 | 0.564 ± 0.043(3) | 0.199 ± 0.031(3) | 0.199 ± 0.031(3) | 0.892 ± 0.168 (5) | NS vs pH 6 + F/I | P<0.01 vs pH/cyclo | NS | P<0.01 |
| pH 6 + cyclo + F/I + AA | 0.650 ± 0.056(5) | NS vs pH 6 | 0.670 ± 0.011(3) | 0.530 ± 0.003(3) | 0.459 ± 0.034(3) | 1.029 ± 0.156 (5) | NS | NS | NS | P<0.01 |
| pH 6 + endo + F/I | 0.146 ± 0.012(3) | NS vs pH 6 | 0.093 ± 0.008(4) | 0.079 ± 0.003(3) | 0.079 ± 0.003(3) | 0.029 ± 0.015(3) | NS | NS | NS | P<0.01 |
| pH 6 + endo + F/I | 0.336 ± 0.022(3) | NS vs pH 6 | 0.293 ± 0.027(4) | 0.149 ± 0.015(3) | 0.149 ± 0.015(3) | 0.074 ± 0.004(5) | NS vs pH 6 + F/I | NS | NS | NS |
| pH 7.4 nod-pip | 0.074 ± 0.004(5) | NS vs cont | 0.084 ± 0.004(5) | NS vs pH 6 | 0.970 ± 0.123(5) | P<0.01 vs pH 6 + F/I | NS | NS | NS | NS |
| pH 6 nod-pip | 0.084 ± 0.004(5) | NS vs pH 6 | 0.084 ± 0.004(5) | NS vs pH 6 | 0.970 ± 0.123(5) | P<0.01 vs pH 6 + F/I | NS | NS | NS | NS |
| pH 6 nod-pip + F/I | 0.970 ± 0.123(5) | P<0.01 vs pH 6 + F/I | NS | NS | NS | NS | NS | NS | NS | NS |
other effects of cyclosporin A, endothal, or nodularin on basal or activated channel activity in either wild-type or mutant hClC-2 were observed (Table III).

DISCUSSION

The cumulative data presented show that both RRAT655 and RGET691 are important to the activation of hClC-2 by PKA phosphorylation at pH 7.4. RGET alone, but not RRAT alone, supported PKA-dependent activation at pH 6.0. However, RRAT did support PKA-dependent activation in the RGET(D) mutant. In addition, these studies show that acid activation of hClC-2 Cl− channel requires phosphorylation of RRAT.

The functional significance of these phosphorylation sites has been investigated using alanine and aspartate substitution of consensus phosphorylation sites (11–14) by site-directed mutagenesis. This is a widely used technique, and loss of activation by protein kinases in such mutants is generally interpreted as a specific modification that does not affect global conformation of the protein. Arachidonic acid, which acts through a separate mechanism to activate hClC-2 Cl− currents, was used to confirm structural integrity of the mutant channels and their presence on the surface of the HEK-293 cells. In addition, the substitution of only one of the two phosphorylation sites generally did not affect the ability to activate at the remaining site, supporting the argument for structural integrity of the mutant channels.

**TABLE IV**

| pH, 7.4          | Control pH, 7.4 | F/I | F/I + AA | Cyclo | Cyclo + F/I | Cyclo + F/I + AA |
|------------------|----------------|-----|---------|-------|------------|-----------------|
| Control pH, 7.4  | 0.204 ± 0.002 (3) | 0.205 ± 0.010 (3) | 0.194 ± 0.009 (3) | 0.193 ± 0.007 (3) | 0.192 ± 0.005 (3) |
| pH, 6            | 0.030 ± 0.003 (3) | 0.033 ± 0.003 (3) | 0.029 ± 0.003 (3) | 0.028 ± 0.003 (3) | 0.027 ± 0.003 (3) |

**TABLE V**

| pH, 7.4          | Control pH, 7.4 | +F/I | +F/I + AA | pH, 6 | pH, 6 + F/I | pH, 6 + F/I + AA |
|------------------|----------------|------|----------|-------|------------|-----------------|
| Control pH, 7.4  | 0.061 ± 0.008 (13) | 0.060 ± 0.014 (5) | 0.059 ± 0.015 (10) | 0.063 ± 0.004 (4) | 0.058 ± 0.024 (4) |
| +F/I             | NS vs. control | p < 0.001 vs. F/I | p < 0.001 vs. F/I | p < 0.001 vs. F/I | p < 0.001 vs. F/I |
| +F/I + AA        | NS vs. control | p < 0.001 vs. F/I | p < 0.001 vs. F/I | p < 0.001 vs. F/I | p < 0.001 vs. F/I |
| pH, 6            | 0.067 ± 0.014 (4) | 0.067 ± 0.014 (4) | 0.067 ± 0.014 (4) | 0.067 ± 0.014 (4) | 0.067 ± 0.014 (4) |
| pH, 6 + F/I      | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    |
| pH, 6 + F/I + AA | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    | NS vs. pH, 6    |

Because there are numerous consensus phosphorylation sites on hClC-2, the site-directed mutagenesis approach would be very tedious, especially because quantitative assessment of the function of the Cl− channels required patch clamp technology. However, based on the initial observation that the human channel contains two phosphorylation sites that are not present in rat ClC-2, which has been repeatedly demonstrated to be insensitive to PKA activation (7, 15), it was reasonable to begin this study by focusing on the sites that are unique to hClC-2.

The loss of the effect of PKA in the double knock-out RRAT(A) plus RGET(A) mutant hClC-2 supported this rationale and greatly limited the number of sites that required detailed study. However, the other potential phosphorylation sites may play a role in regulation of hClC-2 under different conditions.

The RRAT(A) mutant was not responsive to PKA activation at pH 7.4 in the absence of protein phosphatase inhibitors. Wild-type hClC-2 has been previously shown to be responsive to protein phosphatase inhibition (16). Protein phosphatase inhibitors increased PKA activation of the wild-type and the RRAT(A) mutant channel at pH 7.4, demonstrating the importance of control of protein phosphatase activity in PKA activation studies. Thus, the report of lack of PKA activation of 36Cl transport in the human cystic fibrosis (IB-3) cell line containing hClC-2 (8) could be explained in part by the lack of inclusion of phosphatase inhibitors in that study. The same cell line was shown to exhibit low pHi activation in electrophysiological studies. Based on our finding that low pHi activation is dependent upon PKA, it is likely that endogenous PKA activation of hClC-2 occurred in the basal state in those studies (8) showing low pHi activation.

The RRAT site alone is not responsive to PKA activation at pH 6.0 in this model system. PKA itself was active, based on its ability to activate the wild-type and the RRAT(A) mutant hClC-2. Protein phosphatase inhibition was effective because activation of the RRAT(A) mutant containing the RGET site was rescued by protein phosphatase inhibition at pH 7.4 and because cyclosporin A increased forskolin/IBMX-activated wild-type hClC-2 activity at pH 6. Therefore, these results suggest that reduced extracellular pH changed the availability of the RRAT site to protein phosphatases or PKA, perhaps through direct effects on the channel. The RGET(D) mutation also changes the availability of RRAT at reduced pH, suggesting that under some conditions, phosphorylation of RGET may have similar effects.

In support of this suggestion, the RRAT(D) and RGET(D) phosphomimetic mutant hClC-2 channels were constitutively active under basal conditions at both pH 7.4 and 6.0. Some of these could be further activated by PKA through phosphorylation at the RRAT site at pH 7.4 and the RGET and RRAT sites at pH 6.0. The constitutive activity of the RRAT(D) mutant and the inability of PKA to activate the RGET(A) mutant containing RRAT at reduced pH demonstrated that this was
In the presence of cyclosporin A, wild-type, RGET(A), and RRAT(A) channels at pH 7.4 show similar levels of activation by forskolin/IBMX. The difference noted with and without cyclosporin A shows that the wild-type channel has a different phosphatase sensitivity than either of the mutants.

The present study was not designed to consider the physiological significance of any particular protein phosphatase in the regulation of hClC-2 in the HEK-293 model system. Other approaches may be better suited to such studies. For example, a direct interaction between PP1 and rabbit CIC-2 has been demonstrated in a yeast two-hybrid system (18). Endothal has a partial selectivity for PP1, and PP1 inhibition by inclusion of nodularin in the pipette solution increased PKA activation of wild-type hCIC-2 at both pH 7.4 and 6. Additional studies of the action of phosphatases on native hClC-2 channels in cells may be warranted to help evaluate the physiological role of these channels.

This study has identified the residues responsible for PKA activation and PKA-dependent low pH activation of the human CIC-2 channel (RRAT655 and RGET691) and provides some new direction for future evaluation of PKA activation of CIC-2-mediated currents in human tissues. Whereas PKA activation, per se was only 4-fold at pH 7.4 and 2-fold at pH 6.0, there was an 8.6-fold increase at pH 7.4 comparing basal Cl⁻ currents with currents in the presence of cyclosporin A and forskolin/IBMX and a 13-fold increase comparing basal currents at pH 7.4 with currents at pH 6.0 in the presence of cyclosporin A plus forskolin/IBMX (Tables II and III). Several reports of the kinase regulation of CIC-2 in other systems have now appeared (1–5, 16–18), and future studies to investigate the role of PKA activation of CIC-2 in physiological function may benefit from our present studies.

Acknowledgment—We thank Elena Y. Kupert for preparing some of the mutant cDNAs.

REFERENCES

1. Sherry, A. M., Stroffekova, K., Knapp, L. M., Kupert, E. Y., Cuppoletti, J., and Malinowska, D. H. (1997) Am. J. Physiol. 273, C384–C393
2. Tewari, K. P., Malinowska, D. H., Sherry, A. M., and Cuppoletti, J. (2000) Am. J. Physiol. 279, C40–C50
3. Stroffekova, K., Kupert, E. Y., Malinowska, D. H., and Cuppoletti, J. (1998) Am. J. Physiol. 275, C1113–C1123
4. Malinowska, D. H., Kupert, E. Y., Bahinski, A., Sherry, A. M., and Cuppoletti, J. (1995) Am. J. Physiol. 269, C191–C200
5. Cuppoletti, J., Tewari, K. P., Sherry, A. M., Kupert, E. Y., and Malinowska, D. H. (2001) Am. J. Physiol. 281, C46–C54
6. Thieram, A., Gruber, S., Pusch, M., and Jentsch, T. J. (1992) Nature 356, 57–60
7. Park, K., Begenisich, T., and Melvin, J. E. (2001) J. Membr. Biol. 182, 31–37
8. Schiewe, E. M., Gid-Soto, L. P., Stafford, D., Carter, M., Elaisdell, C. J., Zeitlin, P. L., Guggino, W. B., and Cutting, G. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3879–3884
9. Baha, Y., Hikuraka, N., Tanohira, N., and Sodeoka, M. (2003) J. Am. Chem. Soc. 125, 9740–9749
10. Brockdorff, J., Nielsen, M., Svejgaard, A., Dabson, P., Rokpe, C., Geisler, C., and Odum, N. (1997) Cytokine 9, 323–328
11. Zhang, B., Tavare, J. M., Ellis, L., and Roth, R. A. (1991) J. Biol. Chem. 266, 990–996
12. Purves, F. C., Spector, D., and Roizman, B. (1991) J. Virol. 65, 5757–5764
13. Elise, K. E., Weisz, D., and Kustu, S. (1998) J. Mol. Biol. 282, 67–78
14. Onkumar, R. V., Darnay, B. G., and Rodwell, V. W. (1994) J. Biol. Chem. 269, 6810–6814
15. Jentsch, T. J., Stein, V., Weisheit, F., and Zděbik, A. A. (2002) Physiol. Rev. 82, 503–568; Correction (2003) Physiol. Rev. 83, 1a
16. Fritz, J. and Edelman, A. (1997) Am. J. Physiol. 272, C778–C786
17. Kajita, H., Omori, K., and Matsuoka, H. (2000) J. Physiol. 523, 313–324
18. Furukawa, T., Ogura, T., Zheng, Y. J., Tsuchiya, H., Nakaya, H., Katayama, Y., and Inagaki, N. (2002) J. Physiol. 540, 883–893