α1 but Not α2 or α3 Isoforms of Na,K-ATPase Are Efficiently Phosphorylated in a Novel Protein Kinase C Motif†

Pascal Béguin,‡ Manuel C. Peitsch,§ and Käthi Geering*,‡

Institut de Pharmacologie et Toxicologie, rue du Bugnon 27, CH-1005 Lausanne, Switzerland, and Glaxo Institute of Molecular Biology, chemin des Aulx 14, CH-1228 Plan-les-Ouates, Switzerland

Received March 1, 1996; Revised Manuscript Received May 8, 1996⊗

ABSTRACT: Protein kinase C (PKC) phosphorylates the catalytic α1 subunit of Na,K-ATPase in purified enzyme preparations and in intact cells. Little is known, however, whether all three known α isoforms are substrates for PKC and whether direct phosphorylation is implicated in the modulation of the transport activity of the different Na,K-ATPase isozymes. In this study, we investigated the structural requirements for PKC phosphorylation of α1, α2, and α3 isoforms of different species after expression in *Xenopus* oocytes. By using a combination of site-directed mutagenesis and computer-assisted protein modeling, we characterized a novel Ser-*X*-His motif which in concert with more distantly located basic residues acts as an efficient substrate for PKC-mediated phosphorylation in the N-terminus of most Na,K-ATPase α1 isoforms. As indicated by controlled proteolysis, α2 isoforms are also phosphorylated in the N-terminus but to a much lower extent than α1 isoforms containing the Ser-*X*-His motif. Phosphorylation and phosphoamino acid analysis of fusion proteins containing the wild-type or mutant N-terminus of α2 reveal that Thr-Thr-Ser-*X*-Asn or Thr-Thr-Ala-*X*-Asn motifs represent weak targets for PKC phosphorylation. Finally, our data suggest that, with the exception of rat α3, all α3 isoforms from other species are not substrates for PKC. On the basis of the phosphorylation efficiency, we may speculate that only α1 but not α2 or α3 isoforms of Na,K-ATPase are likely candidates for regulatory PKC phosphorylation.

Na,K-ATPase, composed of a catalytic α subunit and a glycosylated β subunit, is an ubiquitous cation pump of the plasma membrane which is responsible for the maintenance of the Na^+ and K^+ gradients existing between the intra- and extracellular milieu of animal cells [for review see Horisberger (1994)]. Three α isoforms have been described which show a tissue-specific distribution and certain distinct properties [for review see McDonough et al. (1995)]. To assure a fine adaptation of the transport capacity to changing physiological demands, Na,K-ATPase activity is governed by numerous short- and long-term regulatory mechanisms [for review see Geering (1995), Bertorello and Katz (1993), and McDonough and Farley (1993)]. Several studies document an acute effect of G protein-coupled receptor activation or of direct protein kinase C (PKC) and A (PKA) stimulation on the Na,K-ATPase activity in different tissues [for review and references see Bertorello and Katz (1993)]. Since stimulation of PKA or of PKC sometimes leads to activation and sometimes to inhibition of the enzyme activity, it is so far not clear whether the kinases produce their effect indirectly or directly by phosphorylation of the Na,K pump. On the other hand, *in vitro* (Chibalin et al., 1992; Beguin et al., 1994; Bertorello et al., 1991; Feschenko & Sweadner, 1994; Lowndes et al., 1990) and *in vivo* (Beguin et al., 1994; Middleton et al., 1993; Borghini et al., 1994; Fisone et al., 1994) phosphorylation studies indeed suggest that Na,K-ATPase can act as a substrate for PKC or PKA phosphorylation but nothing is known whether one or the other α isoforms might be a preferential target for protein kinase modulation.

The site of PKA phosphorylation has been mapped to a serine residue in a highly conserved PKA consensus sequence in the C-terminus of the α subunit (Arg-Arg-Asn-Ser) (Beguin et al., 1994; Fisone et al., 1994; Feschenko & Sweadner, 1995). This site is phosphorylated in intact cells in response to stimulation of PKA by forskolin (Beguin et al., 1994) as well as upon agonist activation of G protein-coupled receptors (Beguin et al., 1994). The identification of the site of PKC phosphorylation in the α subunit was more difficult. In a mutational analysis of the α1 subunit of *Bufo marinus* Na,K-ATPase, we showed that none of the putative PKC consensus phosphorylation sites except a serine residue and to a lesser extent a threonine residue in an unconventional positional context in the cytoplasmic N-terminus were phosphorylated by PKC (Beguin et al., 1994). It is well-known that the phosphorylation motifs for PKC are not as clearly defined as those for PKA. However, a survey of a number of proteins with known PKC phosphorylation sites predicts a (K/R)X_{1-2}(S/T) as a conventional PKC motif (Pearson & Kemp, 1991). The location of the phosphorylated serine and threonine residues in the α1 subunit of *Bufo* Na,K-ATPase deviates considerably from these predictions. A positively charged residue is present at position +2 relative to the phosphorylated serine, and it is a histidine residue which so far has never been described in a PKC phosphorylation motif. Most α1 subunits of different species contain the serine/histidine motif, and controlled tryptic cleavage of the N-terminus abolishes their phosphorylation by PKC (Beguin et al., 1994; Feschenko & Sweadner, 1995). On the other hand, the N-termini of the α2 and α3 isoforms of Na,K-ATPase lack these residues at

† This study was supported by grants from the Swiss National Fund for Scientific Research, No. 31-33676.92 and 31-42954.95.

* Corresponding author.
‡ Institut de Pharmacologie et Toxicologie.
§ Glaxo Institute of Molecular Biology.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

S0006-2960(96)00516-8 CCC: $12.00

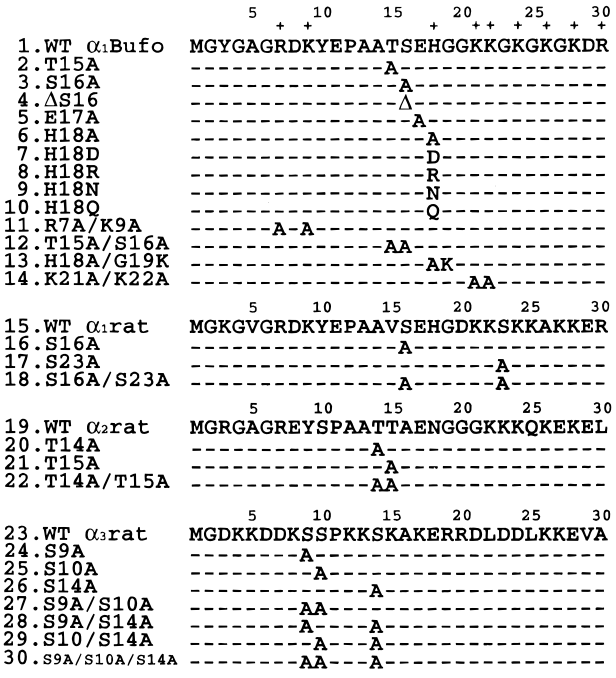

FIGURE 1: Deletion and point mutations introduced in the N-terminus of α subunits of Na,K-ATPase. The amino acid sequence of the most N-terminal amino acids of the wild-type *Bufo* α1 (1−15), rat α1 (16−19), rat α2 (20−23), and rat α3 (24−31) and the introduced amino acid changes are shown. In wild-type *Bufo* α1, the positive charges are marked with a +. Δ indicates a deletion.

the corresponding position and contain instead other serine and/or threonine residues located or not in conventional PKC phosphorylation motifs.

In this study, we have tried to get more insight into the question of whether Na,K-ATPase isozymes might be targets for regulatory phosporylation by PKC. For this purpose, we characterized the novel PKC site in the α1 N-terminus by site-directed mutagenesis combined with computer-assisted modeling of the interaction between the α1 N-terminus and PKC. Furthermore, we investigated the ability of α2 and α3 isozymes to become phosphorylated by PKC. Our data indicate that only the α1 but not the α2 or the α3 isoform is a good sustrate for PKC phosphorylation and in consequence a canditate for regulatory phosphorylation.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The introduction of deletion and single- or double-point mutations into the cDNA of *B. marinus* α1 (Jaisser et al., 1992) rat α1, α2, and α3 (Shull et al., 1986a) (Figure 1) was performed by using the polymerase chain reaction (PCR) method of Nelson and Long (1989). For the *Bufo* mutants, a linearized pSD3 vector (Good et al., 1988) containing an α1 cDNA (pSD3α) was used as a template. The mutants T15A, S16A, and T15/S16A were prepared as described (Beguin et al., 1994). For the mutants H18A, E17A, H18D, H18N, and H18Q (see Figure 1), a DNA fragment was first amplified by PCR between the mutated sense oligonucleotide $G^{40}-C^{60}$ and the antisense oligonucleotide consisting of $G^{705}-G^{723}$ and a primer D of Nelson and Long (1989). The amplified fragments were then used as primers to elongate the inverse DNA strands, and finally the mutated DNA was selectively amplified between a sense oligonucleotide encompassing $T^{2653}-C^{2673}$ of the pSD3 vector and the antisense oligonucleotide D. For the mutant ΔS16, a sense oligonucleotide ^{40}GCCCGCAGCCACCGAACATGGCGGC^{60} was used in the first PCR reaction which permitted the deletion of Ser16. For the preparation of the double mutants R7A/K9A, K21A/K22A, and H18A/G19K (see Figure 1), the sense oligonucleotide of the first PCR reaction contained a double mutation. *Eco*RI and *Xho*I restriction sites were used to introduce the mutated DNA fragment into the wild-type pSD3α.

To increase the translation of foreign cRNA in *Xenopus* oocytes, the 5′-untranslated region (5′-UT) of rat α1, α2, and α3 cDNA (kindly provided by J. Lingrel) was replaced with that of a truncated *Xenopus* α1 cDNA (Burgener-Kairuz et al., 1991). Rat α1 was subcloned into a pSD5 vector containing the modified *Xenopus* 5′UT (pSD5-Xen 5′UT) by digestion with *Sac*I (G^{3158}) and partial digestion with *Nco*I cleaving at the ATG sequence in rat α1. As to rat α2, a DNA fragment (420 nucleotides) was amplified between a mutated sense oligonucleotide $C^{-10}-G^{+10}$ containing an *Nco*I site at the ATG sequence and an antisense oligonucleotide $G^{403}-G^{419}$. The fragment was digested by *Nco*I and subcloned into pSD5-Xen 5′UT. The rat α2 cDNA was removed from its original vector by *Sca*I (T^{25}) and *Nsi*I (A^{4656}) and subcloned into pSD5-Xen 5′UT containing the rat α2 N-terminal fragment by using an *Nsi*I site (present in the vector) and partial digestion with *Sca*I (T^{25}). As to the rat α3, a PCR fragment was generated between a mutated sense oligonucleotide $G^{-9}-A^{+12}$ containing an *Nco*I site at the ATG sequence. The fragment was subcloned into pSD5-Xen 5′UT by using *Nco*I. The rat α3 cDNA was removed from its original vector by *Hin*dIII, partially digested with *Bsm*I (G^{515}), and subcloned into the pSD5-Xen 5′UT containing the rat α3 N-terminal fragment by using *Bsm*I (G^{515}) and *Pma*CI (present in the vector) sites. Linearized pSD5 vectors containing the rat α1, α2, or α3 cDNA modified in the 5′UT were used as templates for rat α mutants. For the rat α1 mutant S16A or S23A (see Figure 1), the DNA was first amplified between the mutated sense oligonucleotide $G^{37}-G^{57}$ or $G^{56}-G^{77}$, respectively, and the antisense oligonucleotide consisting of $G^{310}-G^{334}$ and a VSVG primer. The mutated DNA was selectively amplified between a sense oligonucleotide encompassing $T^{2653}-C^{2673}$ of the pSD5 vector and the antisense VSVG oligonucleotide. The mutated DNA fragments were subcloned into the wild-type pSD5 α1 by using *Nhe*I present in the vector and *Stu*I (G^{184}) after partial digestion. For the preparation of the double mutant S16A/S23A the S16A mutant was used as a template. For the rat α2 mutants T14A, T15A, and T14A/T16A, the DNA was first amplified between the mutated sense oligonucleotide $G^{33}-A^{52}$ and the antisense oligonucleotide consisting of $G^{403}-G^{419}$ and a VSVG primer. The mutated DNA was selectively amplified between a sense oligonucleotide encompassing $T^{2653}-C^{2673}$ of the pSD5 vector and the antisense VSVG oligonucleotide. The mutated DNA fragments were subcloned into the wild-type pSD5α2 by using *Nhe*I sites present in the vector (G^{2757}) and in the rat α2 cDNA (G^{401}). For the rat α3 mutants S9A, S10A, S9A/S10A, or S14A (see Figure 1), the cDNA was first amplified between the mutated sense oligonucleotide $G^{19}-A^{38}$ or $C^{31}-A^{53}$, respectively, and the antisense oligonucleotide $G^{1880}-C^{1895}$ containing a VSVG primer. The mutated cDNA was selectively amplified between a sense oligonucleotide encompassing $T^{2653}-C^{2673}$ of the pSD5

vector and the antisense VSVG oligonucleotide. The mutated fragments were subcloned into the wild-type pSD5α3 vector by using an *Nhe*I site (present in the vector, G^{2757}) and a *Dra*III site (C^{994}). For the preparation of the double mutants S9A/S14A and S10A/S14A or the triple mutant S9A/S10A/S14A, the S14A mutant was used as a template. All PCR-generated fragments were sequenced by dideoxy sequencing (Sanger et al., 1977). cRNAs were prepared from wild-type and mutant α cDNA according to Melton et al. (1984).

Expression of Na,K-ATPase in Xenopus Oocytes. Stage V−VI oocytes were obtained from *Xenopus* females (Noordhoek, Republic of South Africa), treated with collagenase and incubated overnight in modified Barth's medium at 19 °C as described (Geering et al., 1989). Oocytes were then injected with (1) wild type or mutant *Bufo* α1 (8 ng/oocyte) and wild-type *Bufo* β1 (2 ng/oocyte) cRNA, (2) wild-type or mutant rat α1, α2, α3 (8 ng/oocyte) and wild-type rat β1 (1.5 ng/oocyte) (Shull et al., 1986b) cRNA, or (3) wild-type *Xenopus* α1 (5 ng/oocyte) and wild-type *Xenopus* β (0.2 ng/oocyte) (Verrey et al., 1989) cRNA.

To confirm the protein expression, part of the oocytes were incubated in Barth's medium containing 0.6 mCi/mL [^{35}S]-methionine (Amersham) for 72 h before Triton extracts were prepared (Jaunin et al., 1992) and the α subunit was immunoprecipitated (see below). In some instances, yolk-free homogenates or microsomal fractions were prepared from metabolically labeled oocytes expressing rat α1, α2, or α3 and subjected to controlled trypsinolysis (see below). The remaining injected oocytes were incubated for 3 days; yolk-free homogenates were prepared and subjected to phosphorylation (see below).

PKC Phosphorylation of Na,K-ATPase in Homogenates of Xenopus Oocytes. Yolk-free homogenates of *Xenopus* oocytes expressing exogenous α−β complexes were prepared as previously described (Chibalin et al., 1992; Beguin et al., 1994). Briefly, for the preparation of yolk-free homogenates, oocytes were homogenized by 15 strokes in a glass Teflon homogenizer in buffer A containing 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM dithiothreitol, 80 mM sucrose, 0.2 mM ouabain, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5 μg/mL each of antipain, leupeptin, and pepstatin. The samples were centrifuged twice at 100*g* (4 °C, 10 min), and the supernatants were saved.

The phosphorylation reaction in homogenates was done on aliquots corresponding to eight to nine oocytes in the presence of 50 μM [γ-^{32}P]ATP (Amersham), 100 nM phorbol 12-myristate 13-acetate (Sigma, PMA), and 1.5 mM $CaCl_2$ at 25 °C for 30 min. In some instances, samples were subjected to controlled trypsinolysis (see below). The phosphorylation reaction was stopped by addition of SDS (final concentration 3.7%), and the α subunit was immunoprecipitated (see below).

Controlled Trypsinolysis of α Subunits. Rat α1, α2, or α3 subunits expressed in *Xenopus* oocytes and metabolically labeled or phosphorylated in homogenates were subjected to controlled trypsinolysis under conditions which only cleave the cytoplasmic N-terminus of the α subunits. Rat α1, α2, or α3 subunits were digested for 5 min on ice with trypsin at trypsin to protein ratios (w/w) of 0.05, 0.15, or 0.05, respectively, in homogenates, and at trypsin to protein ratios of 0.08, 0.05, or 0.08, respectively, in microsomes, in the presence of deoxycholate at a deoxycholate to protein ratio (w/w) of 0.15. The reaction was stopped by addition of a 5-fold excess (w/w) of soybean trypsin inhibitor (Sigma). After 10 min on ice, SDS was added (final concentration 3.7%), and the α subunits were immunoprecipitated (see below).

Preparation and Phosphorylation of Fusion Proteins Containing Wild-Type or Mutant N-Termini of α Subunits. The fusion proteins containing the N-termini of the *Bufo* wild-type α1 (Gly^6−Lys^{37}) or of the *Bufo* mutants T15A/S16A and H18A were prepared as previously described (Beguin et al., 1994). The fusion proteins containing the N-termini of the wild-type or the mutant rat α1 (Gly^6−Lys^{37}), α2 (Gly^6−Lys^{35} and Met^1−Lys^{35}), and α3 (Asp^6−Lys^{27} and Met^1−Lys^{27}), human α1 (Gly^6−Lys^{37}), and chicken α2 (Gly^6−Lys^{37}) were prepared as follows. The N-termini of rat wild type and mutant α1 were amplified between a sense oligonucleotide CGCGGATCCCGCGGCTGGACGAGAAAGTATG and the antisense oligonucleotide CCGGAATTCGACATTACTTCTTGAGTTCG. The N-termini of rat wild type and mutant α2 were amplified by PCR between a sense oligonucleotide CGCGGATCCCCATGGGACGTGGGGC and the antisense oligonucleotide CCGGAATTCCTTTACTTCTTCAGCTCATC and that of the wild type and mutant α3 between the sense oligonucleotide CGCGGATCCCCATGGGGGACAAAAAAG and the antisense oligonucleotide CCGGAATTCTTACTTCTTGAGGTCATC. The N-termini of the human α1 kindly provided by K. Kawakami (1986) or the chicken α2 kindly provided by K. Takeyasu (1990) were amplified between a sense oligonucleotide CGCGGATCCCGCGGCTGGACGTGATAAGTATG or CGCGGATCCCGCGGCGATGGACGGCCGCGAG, respectively, and the antisense oligonucleotide CCGGAATTCGAAACTTATTTCTTCAGTTCA or CCGGAATTCGACCTACTTCTTCAGCTC, respectively. All sense oligonucleotides contained a *Bam*HI site and all antisense oligonucleotides an *Eco*RI site and a stop codon. The amplified PCR α products were digested by *Bam*HI and *Eco*RI and subcloned into the pGEX-1T vector to generate a glutathione *S*-transferase/α fusion protein. The rat wild-type α1, α2, and α3 N-termini were also subcloned into the pGEX-3X vector. The fusion proteins produced from the two vectors gave similar results in the phosphorylation assays (data not shown). It has been shown that in α1 and α2 isoforms the five first amino acids are missing in the mature enzyme (Jørgensen & Collins, 1986) and thus most fusion proteins were started with the amino acid at position 6. The phosphorylation of these fusion proteins was not different from that starting at Met1 (see Figure 9). The expression and purification of the fusion proteins were done as described by Smith and Johnson (1988). All amplified PCR products were sequenced by dideoxy sequencing (Sanger et al., 1977).

The PKC-dependent phosphorylation assay of the fusion proteins was essentially done as previously described (Beguin et al., 1994) by incubating 6 μg of fusion protein at 30 °C for 15 min in the presence of 15 μM [γ-^{32}P]ATP in a buffer containing 20 mM Hepes (pH 7.4), 13 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 50 μg/mL phosphatidylserine (Sigma), 10 μg/mL 1,2-dioctanoylglycerol (Sigma), 1 mM PMSF, and 0.045 μg of PKC. Phosphoamino acid analysis was performed after separation of the hydrolyzed fusion proteins on one-dimensional thin-layer chromatograms (1600 V, 60 min) essentially as described by Boyle et al. (1991).

Immunoprecipitations of Na,K-ATPase α Subunits. Na,K-ATPase α1 subunits of *Bufo* and α1, α2, or α3 isoforms of rat expressed in oocytes and phosphorylated or metabolically labeled were immunoprecipitated from homogenates or microsomes by an antibody prepared against the purified α subunit of *B. marinus* (Girardet et al., 1981). The immunoprecipitates were separated by SDS−PAGE and revealed by fluorography as previously described (Geering et al., 1987). Quantifications of fluorograms were performed with an analytic program for electrophoretic images (BIO-1D) from Vilber Lourmat (Marne LaVallée, France). In preliminary experiments, we determined the efficiency of immunoprecipitation by the *Bufo* α-antibody of the rat α isoforms by comparing the signal of native and immunoprecipitated α isoforms synthesized in a reticulocyte lysate. The immunoprecipitation efficiency of the *Bufo* α-antibody was 1, 0.82, 0.4, and 0.1 for *Bufo* α1, rat α1, rat α2, and rat α3 subunits, respectively.

Computer-Aided Molecular Modeling. The sequence of bovine protein kinase C-α (Swiss-Prot: KPCA-BOVIN) was compared with all sequences for which a three-dimensional structure is available in the Brookhaven Protein Data Bank (PDB). The database entries with the highest degree of sequence identity [34%; BLAST unlikelihood probability of 10^{-56} (Altschul et al., 1990)] correspond to the crystal structures of the catalytic subunit of the cAMP-dependent protein kinase (PDB entries 1CTP, 1ATP, 2CPK, 1CMK, and 1APM). This degree of sequence identity is sufficient to attempt knowledge-based protein modeling, which we performed by using the automated protein modeling package ProMod (Peitsch, 1996). A similar approach has been used to model the catalytic domain of the PKC βII (Orr & Newton, 1994). We derived a multiple sequence alignment from the optimally superimposed coordinate sets. The sequence of bovine protein kinase C-α was then added to this alignment using the results of pairwise comparisons performed with SIM (Huang & Miller, 1991). The modeling procedure involved the following steps (Peitsch, 1996): (i) the construction of an average framework from the superimposed subunits of all selected entries; (ii) the generation of atomic coordinates derived from the averaged framework by using the multiple sequence alignment described above; (iii) the rebuilding of the altered loops from their stems by structural homology searches through the Brookhaven Data Bank as described by Greer (1990); (iv) the completion of the main chain by using a library of backbone elements (pentapeptides) derived from the best X-ray structures (<2 Å resolution); (v) the reconstitution of lacking side chains and the correction of existing ones using a library of allowed rotamers (Ponder & Richards, 1987). Optimization of bond geometry and relief of unfavorable nonbonded contacts were performed by 30 steps of steepest descent followed by 500 steps of conjugate gradient minimization using the CHARMM package (Brooks et al., 1983) with the PARAM19 parameter set. The 3D−1D profile matching procedure of Luethy et al. (1992) was used to assess the quality of the model.

The peptide substrate corresponding to the N-terminus of the *Bufo* α1 subunit was modeled after the structure of the peptide inhibitor of protein kinase A: T-T-Y-A-D-F-I-A-S-G-R-T-G-R-R-N-A-I-H-D (A represents the alanine residue at the position of the phosphorylated serine or threonine) described in the PDB entry 1ATP (Zheng et al., 1993). The peptide−enzyme complex was further refined by 1000 steps of conjugate gradient energy minimization.

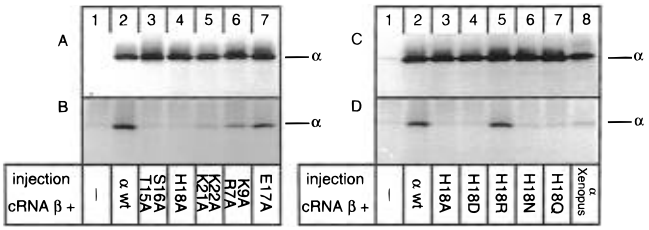

FIGURE 2: Effect of mutations in basic residues of the N-terminus on PKC-mediated phosphorylation of the *Bufo* α1 subunit in *Xenopus* homogenates. *Xenopus* oocytes were injected with *Bufo* β1 cRNA alone (lane 1) or with *Bufo* β1 cRNA plus *Bufo* wild-type (α wt) (lane 2) or mutant α cRNA (lanes 3−7) or with *Xenopus* wild-type α1 and β1 cRNA (lane 8) as described in Experimental Procedures. (A and C) Cellular expression of wild-type or mutant *Bufo* α1 subunits. After cRNA injection, oocytes were metabolically labeled with ^{35}S-methionine and extracted with Triton X-100 as described in Experimental Procedures. Shown are autoradiograms of immunoprecipitated α subunits. (B and D) PKC-mediated phosphorylation of wild-type or mutant *Bufo* α1 subunits. After cRNA injection, oocytes from the same batch as used in the experiments shown in (A) and (C) were incubated for 3 days before preparation of homogenates. The phosphorylation reaction was done as described in Experimental Procedures in the presence of 100 nM PMA. Shown are autoradiograms of immunoprecipitated α subunits. (A) and (B) or (C) and (D), respectively, show one out of two similar experiments.

RESULTS

Characterization of the PKC Phosphorylation Motif in α1 Isoforms. We have previously reported that PKC phosphorylates the α1 subunit of *Bufo* Na,K-ATPase on Ser16 and on Thr15 which are not located in a known PKC consensus sequence (Beguin et al., 1994). To better define the structural requirements for PKC phosphorylation in general and in the Na,K-ATPase α1 isoforms in particular, we prepared a series of mutants affected in the N-terminus of the *Bufo* α1 subunits, expressed them in *Xenopus* oocytes together with β subunits, and tested their ability to become phosphorylated in homogenates after stimulation of the endogenous, oocyte PKC with PMA. We have previously shown that PKC phosphorylation of α subunits is similar in this test system than in intact cells (Beguin et al., 1994, 1996).

In all proteins which are known to be phosphorylated by PKC, one or several positively charged lysine and/or arginine residues are present in the close proximity of the phosphorylated serine or threonine residues (Pearson & Kemp, 1991) which are likely to be important for the interaction with the active site in PKC. Since this is not the case in α1 isoforms of Na,K-ATPase, we first looked at the importance for PKC-mediated phosphorylation of the positively charged histidine at position +2 relative to the phosphorylated serine as well as of the more distantly located lysine and arginine clusters present in the N-terminus (see Figure 1). Biosynthetic labeling with [^{35}S]methionine of *Xenopus* oocytes injected with *Bufo* α and β cRNA showed that the wild-type α subunit and several mutants affected in positively charged residues were expressed to a similar extent and well above the level of endogenous, oocyte α subunits (Figure 2A). As previously reported (Beguin et al., 1994), PMA-mediated phosphorylation of the wild-type *Bufo* α subunit gave a signal about 10−15-fold over background levels (Figure 2B,

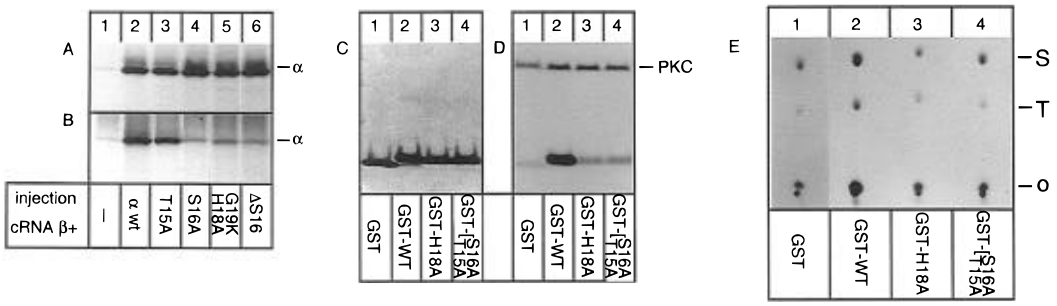

FIGURE 3: Effect of charge displacement on PKC-mediated phosphorylation of Ser16 and Thr15 in the *Bufo* α1 subunit. (A) Cellular expression of wild-type and mutant *Bufo* α1 subunits. *Xenopus* oocytes were injected with *Bufo* β1 cRNA alone (lane 1) or with *Bufo* β1 cRNA plus *Bufo* wild-type (α wt) (lane 2) or mutant α cRNA (lanes 3−7), metabolically labeled with [^{35}S]methionine, and extracted with Triton X-100 as described in Experimental Procedures. Shown are autoradiograms of immunoprecipitated α subunits. (B) PKC-mediated phosphorylation of wild-type or mutant *Bufo* α1 subunits. After cRNA injection, oocytes from the same batch as used in the experiments shown in (A) were incubated for 3 days before preparation of homogenates. The phosphorylation reaction was done as described in Experimental Procedures in the presence of 100 nM PMA. Shown are autoradiograms of immunoprecipitated α subunits. One out of two similar experiments is shown. (C and D) PKC-mediated phosphorylation of fusion proteins containing the wild-type or mutated N-terminus of the *Bufo* α1 subunit. Fusion proteins containing glutathione *S*-transferase (GST, lane 1) and the wild-type (GST-WT, lane 2) or mutated (lanes 3 and 4) N-terminus of the *Bufo* α subunit were prepared and subjected to a phoshorylation reaction in the presence of purified PKC as described in Experimental Procedures. The phosphorylated products were resolved by SDS−PAGE, stained with Coomassie Blue (C), and revealed by autoradiography (D). PKC = autophosphorylated protein kinase C. (E) Phosphoamino acid analysis of wild-type and mutant fusion proteins. Phosphorylated fusion proteins shown in (C) and (D) were cut out of the gel and subjected to phosphoamino acid analysis by one-dimensional thin-layer chromatography as described in Experimental Procedures. S and T indicate the positions of cold phosphoserine and phosphothreonine, respectively. o represents the origin of the sample application. One out of two similar experiments is shown.

compare lane 2 to lane 1) while the phosphorylation of the α subunit mutated at Ser16 and Thr15 (S16A/T15A) was abolished (compare lane 3 to lane 1). The serine−threonine phosphorylation of the α subunit was abolished after replacement of His18 by alanine (H18A, lane 4) and reduced by 83% (average of two experiments) or 36% (average of two experiments) after replacement of Lys22 and Lys21 (K22A/K21A, lane 5) or of Lys9 and Arg7 (K9A/R7A, lane 6), respectively. In contrast, mutations in the negatively charged glutamic acid (E17A) at position +1 relative to the phosphorylated serine residue did not significantly affect the phosphorylation of the α subunit (lane 7). Thus, these data indicate that the presence of basic residues, of the histidine residue at position +2, on the one hand, and of the lysine and arginine residues at positions −7 and −9 or +5 and +6, on the other hand, is crucial for an efficient serine phosphorylation of the α subunit of *Bufo* Na,K-ATPase by PKC.

To further investigate the role of His18 and in particular to distinguish whether its electrostatic or hydrogen-bonding capacity is implicated in the phosphorylation event, we replaced His18 either by another positively charged amino acid, an arginine (H18R), or by a negatively charged amino acid, an aspartic acid (H18D), or by polar, uncharged amino acids, asparagine or glutamine (H18N, H18Q). All mutants were similarly expressed in oocytes as compared to the wild-type α subunit (Figure 2C). Significantly, the phosphorylation of the α subunit was only preserved in the H18R mutant which maintains a positively charged residue at position 18 (Figure 2D, compare lane 5 to lane 2). In contrast, the phosphorylation was abolished in the H18D mutant (compare lane 4 to lane 1) and decreased by 90% and 96% in the H18N and H18Q mutants, respectively (average of two experiments) (compare lanes 6 and 7 to lane 2). Thus, it appears that the electrostatic bonding capacity of His18 is important for an efficient phosphorylation of the α1 subunit. This result is also supported by the fact that the phosphorylation of *Xenopus* α1 subunit, in which His18 is replaced by a glutamine residue (see Figure 5), is only about 15% of that of the *Bufo* α1 subunit (Beguin et al., 1994; Figure 2, lane 8). In addition, the data indicate that histidine and arginine are equally effective in promoting PKC-mediated phosphorylation.

The α1 subunit of *Bufo* Na,K-ATPase is mainly phosphorylated on Ser16 and to a lesser extent on Thr15 (Beguin et al., 1994). The phosphorylation signal is decreased by about 10% in the T15A mutant (Figure 3B, lane 3) and by about 90% in the S16A mutant (lane 4) compared to that of the wild-type α subunit (lane 2). The question arose whether the position at +2 of the positively charged histidine residue is important for the phosphorylation of Ser16 and/or determines the differential phosphorylation behavior of Ser16 and Thr15. To assess these questions, we first shifted the basic residue from position +2 to position +3 relative to Ser16 by replacing Gly19 by a lysine residue in the H18A mutant (G19K/H18A). The phosphorylation of the G19K/H18A mutant was about 75% reduced compared to that of the wild type α subunit (compare lane 5 to lane 2), indicating that the distance of the positively charged residue from Ser16 is essential for its optimal phosphorylation. Furthermore, the phosphorylation of a mutant in which Ser16 was deleted and in which the positively charged histidine residue was shifted to position +2 relative to Thr15 was not significantly increased compared to that of the S16A mutant (ΔS16, compare lane 6 to lane 4), suggesting that serine cannot be replaced by a threonine residue and/or that the Ser16 deletion alters the position of more distantly located positively charged residues which are important for Thr15 phosphorylation.

We have previously shown that fusion proteins containing the N-terminus of α subunits are good tools to identify phosphorylation sites (Beguin et al., 1994). Mutational studies on fusion proteins provide the same results on the nature of the phosphorylation sites as those performed on native Na,K-ATPase. To test for the importance of His18 for Thr15 phosphorylation, we therefore prepared fusion proteins between glutathione *S*-transferase (GST) and the N-terminus of the *Bufo* α1 subunit mutated at His18 and

checked by phosphoamino acid analysis whether the mutated N-terminus was residually phosphorylated or not on Thr15 after incubation with purified PKC. Panels C and D of Figure 3 show the Coomassie staining and the phosphorylation patterns, respectively, of the GST and the GST fusion proteins. GST alone showed a certain basal phosphorylation after incubation with PKC (Figure 3D, lane 1) which was mainly due to serine phosphorylation (ratio of serine to threonine phosphorylation close to 4) (Figure 3E, lane 1). The phosphorylation signal increased by about 6-fold (average of five experiments) in the fusion protein containing the wild-type N-terminus of *Bufo* α1 (GST-WT; Figure 3D, lane 2) but did not significantly change and was similar in the mutated fusion proteins GST-H18A (lane 3) and GST-S16A/T15A (lane 4). The phosphoamino acid analysis clearly showed that not only the serine signal but also the threonine signal is reduced to that of GST (lane 1) in GST-S16A/T15A (lane 4) as well as in GST-H18A (lane 3). This result indicates that His18 is important not only for the Ser16 but also for the Thr15 phosphorylation.

Computer Modeling of the Interaction of the N-Terminus of the Na,K-ATPase α1 Subunit with PKC. To better understand the nature of the interaction between the N-terminus of the Na,K-ATPase α1 subunit and PKC, we built molecular models of both the N-terminal part of the α1 subunit and the bovine protein kinase C using knowledge-based protein modeling methods. This model was not used to get detailed information on residue−residue interaction but rather to guide the discussion and the interpretation of the mutation studies. The catalytic subunit of the cAMP-dependent protein kinase (PKA) was used to build this molecular model. The N-terminus of the Na,K-ATPase α1 subunit was modeled after the peptide inhibitor of PKA which was cocrystalized with the enzyme (PDB entry 1ATP; Zheng et al., 1993).

Using the 3D−1D profile matching procedure of Luethy et al. (1992), the PKC model yielded an average score of 0.59. Furthermore, the two profiles did not present marked differences (data not shown), indicating that the PKC model is of reasonable quality.

The peptide-binding domain in PKC is a large region spread throughout the larger lobe and comprises numerous putative contact sites. In particular, a striking feature of PKC is the presence of a large number of acidic residues in two areas (Figure 4). First, the loop linking helices B and C (Asp379 to Asp380) contains three aspartates in a row, and second, the loop linking helices F and G (Asp539 to Glu545) consists of alternating aspartates and glutamates interrupted by a glycine (Gly540). It seems reasonable that these regions largely contribute to the binding of peptides to the enzyme and determine the substrate specificity. Indeed, PKA only bears one negative charge in the same two regions. The C-terminal region (following helix J) may also contribute to the substrate specificity since, in this region, PKA is highly negatively charged between Asp238 and Glu334 (six acidic residues) while PKC bears only one aspartate in the corresponding segment (residues 627−634).

The main residue phosphorylated by PKC in the N-terminus of the *Bufo* Na,K-ATPase α1 subunit is Ser16. During the modeling of the substrate, we aligned this residue with Ala21 in the PKA inhibitor structure. The position of this particular alanine residue, relative to the active site of PKA, corresponds to the one a serine or a threonine has to take in order to be phosphorylated (Kemp et al., 1994). The resulting spacial localization of His18 allows it to interact electrostatically with Asp378 and possibly with both Asp379 and Asp380, depending on its side-chain rotamer, of our PKC model. This strong interaction seems to be essential in our substrate since the replacement of His18 by either alanine or aspartic acid, in contrast to arginine, abolishes its phosphorylation (see above). These data demonstrate that position +2 relative to the phosphorylated serine must be occupied by either a histidine or a basic residue.

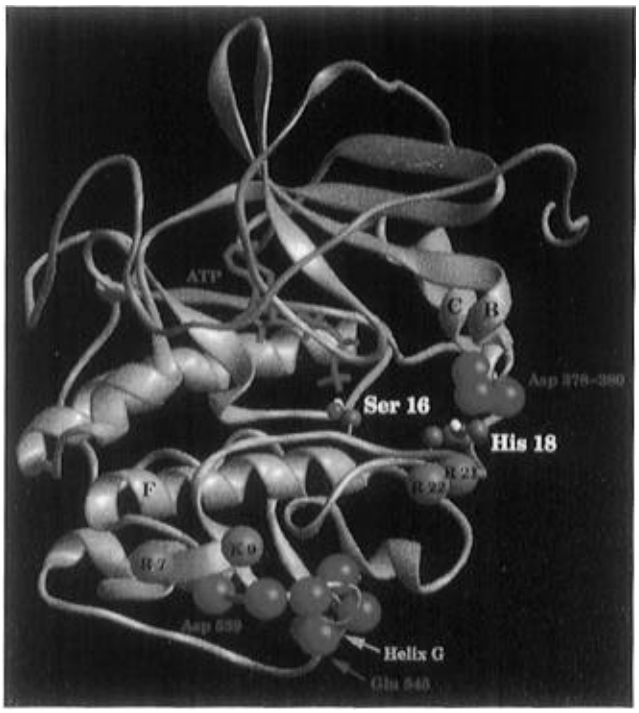

FIGURE 4: Molecular model of protein kinase C interacting with the 22 N-terminal amino acid residues of the α1 subunit of *Bufo* Na,K-ATPase. PKC is represented as a gray ribbon and the substrate is in orange. The ATP structure is in magenta. The key Ser16 (target residue for phosphorylation) and His18 (essential contact site) in the substrate are shown as ball and stick models (green, C; red, O; violet, N; white, H). The relative positions of the four basic residues in the substrate and of the acidic amino acids in PKC are shown by blue and red spheres, respectively. To facilitate the model description, all residues are numbered according to the Swiss-Prot entry KPCA-BOVIN, while the loops and strands are named according to Taylor et al. (1992, 1993).

The basic residues which precede (Arg7 and Lys9) or follow (Lys21 and Lys22) Ser16 at a larger distance than His18 in our substrate are most likely involved in electrostatic interactions with the negatively charged C−B and F−G loops in the PKC. The F−G loop has already been proposed by Orr and Newton (1994) to be involved in substrate binding, based on a molecular model. Though a definite assignment cannot be made on the basis of a molecular model, it is probable that the N-terminally located, basic residues in the substrate will interact with the loop linking the F and G helices. On the other hand, the C-terminally located, basic residues may contact either of the two regions.

PKC-Mediated Phosphorylation of Rat α1 Isoforms. As previously shown by controlled trypsinolysis (Beguin et al., 1994), not only the α1 subunits of purified *Bufo* and *Xenopus* Na,K-ATPase but also that of sheep, rabbit, and duck are exclusively phosphorylated by PKC in the cytoplasmic N-terminal tail. All known α1 isoforms contain either the

```
                                                     tryptic site 2
           1        10          20          30       |  40
                 + +         +   ++   + + + +       ++v
Buf NKα1  MGYGAGRDKYEPAA.TSEHGGKK..GKGKGKDRDMEELKKEVTME
Xen NKα1  MGYGAGRDKYEPAA.TSEQGGKKKKGKGKGKEKDMDELKKEVTME
Chi NKα1  MGKGAGRDKYEPTA.TSEHGTKK...KKAK.ERDMDELKKEVSMD
Rat NKα1  MGKGVGRDKYEPAA.VSEHGDKK..SKKAKKERDMDELKKEVSMD
Hum NKα1  MGKGVGRDKYEPAA.VSEQGDKK..GKKGKKDRKMKELKKEVSMD
           1         10         20            30      40
Chi NKα2  M...DGRE.YSPAATTSENGGGRRKQ....KEKELDELKKEVNLD
Rat NKα2  MGRGAGRE.YSPAATTAENGGGKKKQ....KEKELDELKKEVAMD
Hum NKα2  MGRGAGRE.YSPAATTAENGGGKKKQ....KEKELDELKKEVAMD
           1         10                  20         30
Chi NKα3  MG.DKG.EKESP.........KK..GKGK...RDLDDLKKEVAMT
Rat NKα3  MG.DKKDDKSSP.........KK..SKAKER.RDLDDLKKEVAMT
Hum NKα3  MG.DKKDDKDSP.........KK..NKGKER.RDLDDLKKEVAMT
```

FIGURE 5: Alignment of N-terminal amino acid sequences of Na,K-ATPase α subunits. Shown are amino acid sequences of *B. marinus*, *Xenopus laevis*, chicken, rat, and human α1 and of chicken, rat and human α2 and α3 subunits [for references see Horisberger (1994)]. Gaps were introduced by hand to optimize the alignment of homologous residues. The positions of positive amino acids refer to the *Bufo* α1 subunit. The numbers refer to amino acids of *Bufo* α1, rat α2, and rat α3, respectively. The position of the tryptic cleavage site 2 according to Jørgensen and Farley (1988) is indicated. The putative motifs for PKC-mediated phosphorylation in *Bufo* and rat α1 and in rat α2 and α3 are underlined. The identified phosphorylated serine and/or threonine residues are in bold. For further explanations see text.

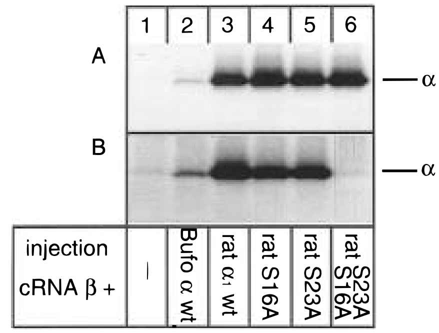

FIGURE 6: PKC-mediated phosphorylation of α1 isoforms of rat Na,K-ATPase and analysis of the phosphorylation motif. *Xenopus* oocytes were injected with rat β1 cRNA alone (lane 1) or with *Bufo* (lane 2) or rat (lane 3−6) β1 cRNA plus *Bufo* (lane 2) or rat wild-type (lane 3) or rat mutant (lanes 4−6) α1 cRNA. (A) Cellular expression of wild-type and mutant rat α1 subunits. After cRNA injection, oocytes were metabolically labeled with [^{35}S]methionine and extracted with Triton X-100 as described in Experimental Procedures. Shown are autoradiograms of immunoprecipitated α subunits. (B) PKC-mediated phosphorylation of wild-type or mutant rat α1 subunits. After cRNA injection, oocytes from the same batch as used in the experiments shown in (A) were incubated for 3 days before preparation of homogenates. The phosphorylation reaction was done as described in Experimental Procedures in the presence of 100 nM PMA. Shown are autoradiograms of immunoprecipitated α subunits. One out of two similar experiments is shown.

Ser-Glu-His or the Ser-Glu-Gln motif in the N-terminus (Figure 5) indicating that the observed PKC-mediated phosphorylation at Ser16 in the *Bufo* and *Xenopus* α1 subunit is of general importance. A sequence survey reveals that α1 subunits of rat (Figure 5) and of the white sucker *Catostomus commersoni* are atypical in that they have additional serine residues in the N-terminus which are located in more conventional PKC consensus sequences and which could serve as phosphorylation sites. To check for the relative importance of Ser16 and Ser23 (contained in the sequence Lys-Lys-Ser-Lys-Lys) in PKC-mediated phosphorylation of the rat α1 subunit, we mutated these residues individually or together and tested the phosphorylation of the mutants after expression in *Xenopus* oocytes. The rat wild-type and mutant α1 subunits were similarly expressed in oocytes and well recognized by a *Bufo* α-antibody used for immunoprecipitations (Figure 6A, lanes 3−6; see Experimental Procedures). By comparison of the expression (lanes 2 and 3) and phosphorylation levels (Figure 6B, lanes 2 and 3), it can be estimated that the rat wild-type α1 was phosphorylated to an extent similar to that of the *Bufo* α1 subunit (three experiments). As to the rat α1 mutants, the PKC-mediated phosphorylation of both single mutants, S23A (lane 4) and S16A (lane 5), was decreased by about 25% (average of two experiments) compared to that of the rat wild-type α1 subunit (lane 3). These results differ from experimental data obtained by sequence analysis of phosphorylated, purified enzyme preparations of rat α1 subunits (Feschenko & Sweadner, 1995) in which Ser16 and Ser23 phosphorylation accounted for 25% and 75%, respectively, of the PKC phosphorylation. A possible explanation for these differences is that mutation of Ser23 favors the phosphorylation of Ser16. Despite the uncertainty of the relative phosphorylation of Ser16 and Ser23, it is clear that they are the only amino acid residues which contribute to PKC phosphorylation of rat α1 subunits. Indeed, phosphorylation was completely abolished in the double mutant S23A/S16A (compare lane 6 to lane 1). This latter result was also confirmed by phosphorylation assays on GST fusion proteins containing the wild-type or mutant N-terminus of the rat α1 subunit (data not shown).

Altogether, the results obtained with rat α1 subunits confirm the importance of Ser16 in the PKC-mediated phosphorylation of the Na,K-ATPase α1 isoforms but they also indicate that other sites in the N-terminus of more conventional nature might be used, if available. Whether the phosphorylation of two sites indeed leads to an increased overall phosphorylation of the rat α1 isoform compared to that of the other α1 subunits is actually not clear. In contrast to our results, Feschenko and Sweadner (1995) observed that the α1 subunit of purified rat Na,K-ATPase is more efficiently phosphorylated than enzymes which only contain the Ser-X-His motif. The reason for these discrepancies is not known, but the results suggest that the conformational state of the N-terminus might influence the phosphorylation efficiency.

PKC-Mediated Phosphorylation of α2 and α3 Isoforms. So far, it is not known whether α2 and α3 isoforms of Na,K-ATPase are phosphorylated by PKC and if so whether phosphorylation occurs in the N-terminus as in the α1 isoform. Sequence comparison shows a great variation in the N-termini of α1, α2, and α3 isoforms (see Figure 5). In both the α2 and α3 N-termini the Ser-Glu-His(Gln) motif is missing. Instead, the α2 N-terminus of rat and man contains two conserved amino acid stretches with serine or threonine residues. Of these, ^{7}Arg-Glu-Tyr-Ser^{10} but not ^{14}Thr-Thr-Ala-Glu-Asn^{18} represents a putative PKC motif. On the other hand, the α3 isoforms bear one to three serine residues within conventional PKC sites (see Figure 5). To check for an N-terminal PKC-mediated phosphorylation, we expressed rat α1, α2, or α3 isoforms in *Xenopus* oocytes and, after PKC-mediated phosphorylation in homogenates, subjected them to a controlled trypsinolysis assay which selectively cleaves the N-terminus of the α subunit (Beguin et al., 1994). All three rat α isoforms are well expressed in oocytes (Figure 7A, lanes 2, 4, and 6). The lower signal

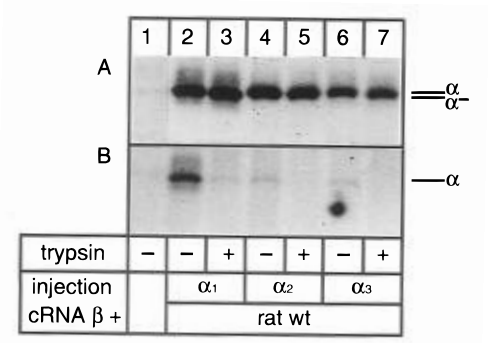

FIGURE 7: PKC-mediated phosphorylation of the N-terminus of α2 and α3 isoforms of rat Na,K-ATPase in *Xenopus* oocyte homogenates. (A) Controlled trypsinolysis of metabolically labeled rat α isoforms. *Xenopus* oocytes were injected with rat β1 cRNA alone (lane 1), or with rat β1 cRNA plus rat α1 (lanes 2 and 3), rat α2 (lanes 4 and 5), or rat α3 (lanes 6 and 7) cRNA. After cRNA injection, oocytes were metabolically labeled with [^{35}S]methionine before preparation of homogenates. Aliquots of homogenates were incubated with (lanes 3, 5, and 7) or without (lanes 1, 2, 4 and 6) trypsin for 5 min at 4 °C as described in Experimental Procedures before immunoprecipitation. Shown are autoradiograms of immunoprecipitated α subunits. α$^-$ indicates the position of the trypsinized α subunit lacking the N-terminus (lanes 3, 5, and 7). (B) Controlled trypsinolysis of rat α isoforms phosphorylated in *Xenopus* homogenates in response to PKC stimulation. After cRNA injection, oocytes from the same batch as used in the experiments shown in (A) were incubated for 3 days before preparation of homogenates. The phosphorylation reaction was done as described in Experimental Procedures in the presence of 100 nM PMA. After phosphorylation, aliquots of homogenates were incubated with (lanes 3, 5, and 7) or without (lanes 1, 2, 4, and 6) trypsin under the same conditions as for [^{35}S]methionine-labeled samples shown in (A). The faint phosphorylated band observed in lane 2 is due to phosphorylation of undigested α1 subunits which do not show a shift in the molecular mass. Shown are autoradiograms of immunoprecipitated α subunits. One out of two similar experiments is shown.

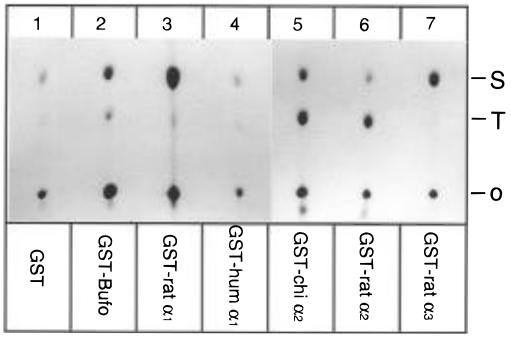

FIGURE 8: Phosphoamino acid analysis of fusion proteins containing the N-terminus of α1, α2, or α3 isoforms. Fusion proteins between glutathione *S*-transferase (GST, lane 1) and the N-terminus of *Bufo* α1 (lane 2), rat α1 (lane 3), human α1 (lane 4), chicken α2 (lane 5), rat α2 (lane 6), or rat α3 (lane 7) were prepared, subjected to a phosphorylation reaction in the presence of purified PKC, and subjected to phosphoamino acid analysis as described in Experimental Procedures. S, T, and o are as in Figure 3. One out of two similar experiments is shown.

observed with α3 isoforms (lane 6) is probably mainly due to a less efficient recognition by the *Bufo* α-antibody used to immunoprecipitate the rat α isoforms (see Experimental Procedures). Rat α2 and α3 isoforms became phosphorylated in response to PKC stimulation but to a much lower extent than α1 (Figure 7B, compare lanes 4 and 6 to lane 2). By taking into account the expression level (and/or the antibody recognition), the efficiency of phosphorylation of rat α3 and α2 is about 25% and 10% of that of rat α1 (average of three experiments). Selective, tryptic cleavage of the N-terminus reflected by the small shift in the molecular mass of the biosynthetically labeled proteins (α$^-$, Figure 7A, lanes 3, 5, and 7) abolished the phosphorylation signal in all three α isoforms (Figure 7B, lanes 3, 5, and 7). Similar results were obtained with rat α isoforms phosphorylated by purified PKC in microsomes of cRNA-injected oocytes (data not shown).

These data indicated that, as α1 isoforms, α2 and α3 isoforms of rat are phosphorylated in the N-terminus, but in contrast to α1, they represent poor substrates for PKC. To further characterize PKC phosphorylation of α2 and α3 isoforms, we identified the nature of the phosphorylated amino acid in GST fusion proteins containing the N-termini of α1, α2, or α2 isoforms of different species. Phosphoamino acid analysis revealed that, in agreement with the previous findings on the native α subunits, the N-terminus of *Bufo* α1 is phosphorylated mainly on serine and to a lesser extent on threonine (Figure 8C, lane 2) and that of rat α1 only on serine residues (lane 3). In the human α1 N-terminus neither serine nor threonine residues were visibly phosphorylated (lane 4). As to α2 and α3 isoforms, the N-terminus of chicken α2 (lane 5) was phosphorylated on both serine and threonine, that of rat α2 (lane 6) only on threonine, and that of rat α3 (lane 7) only on serine residues. These data confirm that the Thr-Ser-Glu-His and the Ser-Glu-His motifs in *Bufo* and rat α1 N-termini but not the Ser-Glu-Gln found in human and *Xenopus* α1 N-termini are efficient substrates for PKC phosphorylation. In addition, the data suggest that rat and chicken α2 are mainly phosphorylated in the ^{14}Thr-Thr-Ala-Glu-Asn^{18} (see Figure 5) and the ^{11}Thr-Thr-Ser-Glu-Asn^{15} sequence, respectively. Finally, the rat α3 N-terminus is obviously phosphorylated on serine residues in the ^{8}Lys-Ser-Ser-Pro-Lys-Lys-Ser-Lys-Ala-Lys^{17} sequence.

Confirmation of these latter results was obtained by mutational analysis. Unfortunately, due to the low phosphorylation efficiency in *Xenopus* homogenates, no conclusive results could be obtained with mutated, native α2 and α3 isoforms. However, phosphoamino acid analysis of GST fusion proteins containing the N-terminus of the rat α2 isoform mutated in Thr15 and/or Thr14 showed that a double GST T15A/T14A mutant abolished phosphorylation at threonine residues to the basal GST phosphorylation level (Figure 9A, compare lane 5 to lane 1) and that both Thr14 and Thr15 accounted for about 50% (average of two experiments) of the threonine phosphorylation of the wild-type α2 N-terminus (compare lanes 3 and 4 to lane 2). Thus, phosphorylation studies on fusion proteins and native α2 isoforms indicate that the N-terminus of the rat α2 is a possible, but poor substrate for PKC phosphorylation and that the unusual ^{14}Thr-Thr-Ala-Glu-Asn^{18} is responsible for this phosphorylation.

To identify the phosphorylated serine residue in the N-terminus of the α3 isoform, we tested the phosphorylation of GST fusion proteins containing the rat α3 wild-type N-terminus or the N-terminus mutated in Ser9, Ser10, and/or Ser14 (Figure 9B,C). Since it is not known whether in the α3 isoform similar to the α1 and α2 isoforms (Jørgensen & Collins, 1986) the first five amino acids are missing, we compared the phosphorylation of fusion proteins starting with Asp6 or with Met1. Compared to GST (Figure 9C, lane 1), PKC-mediated phosphorylation increased by 5-fold (average of four experiments) in both fusion proteins (lanes 2 and 3),

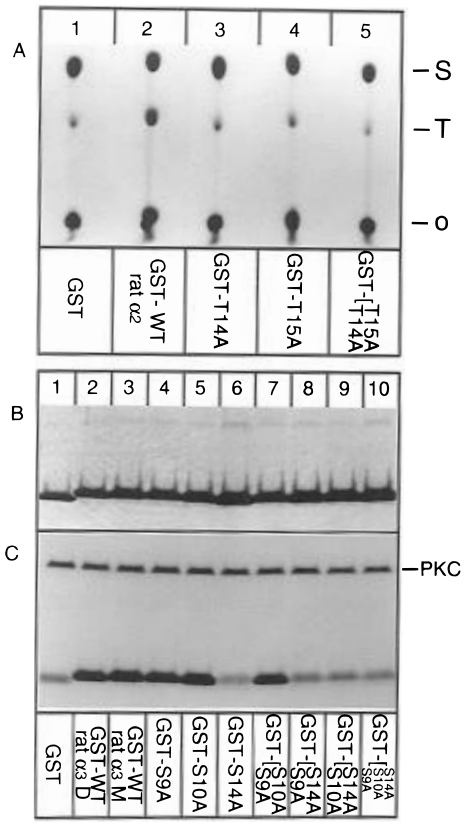

FIGURE 9: Analysis of PKC phosphorylation motifs in the N-terminus of the α2 and α3 isoforms. (A) Phosphoamino acid analysis of fusion proteins containing the wild-type or mutated N-terminus of the rat α2 isoform. Fusion proteins between glutathione *S*-transferase (GST) and the wild-type (GST-WT rat α2) or the mutated N-terminus of rat α2 were subjected to a phosphorylation reaction in the presence of purified PKC as described in Experimental Procedures. The phosphorylated products were resolved by SDS−PAGE, revealed by autoradiography, and subjected to phosphoamino acid analysis. S, T, and o are as in Figure 3. (B and C) PKC-mediated phosphorylation of fusion proteins containing the wild-type or mutated N-terminus of the rat α3 isoform. Fusion proteins between glutathione *S*-transferase (GST, lane 1) and the wild-type N-terminus of rat α3 starting at Asp^5 (lane 2) or Met^1 (lane 3), or the mutated N-terminus of rat α3 starting at Met^1 (lanes 4−10), were prepared and subjected to a phosphorylation reaction in the presence of purified PKC as described in Experimental Procedures. The phosphorylated products were resolved by SDS−PAGE, stained with Coomassie Blue (B), and revealed by autoradiography (C). PKC = autophosphorylated protein kinase C.

indicating that the presence or absence of the most N-terminal amino acids does not influence the overall phosphorylation of the α3 N-terminus. Significantly, the PKC-mediated phosphorylation of single or double mutants affected in Ser9 and Ser10 was not decreased compared to that of the wild type α3 fusion protein (Figure 9C, compare lanes 4, 5, and 7 to lane 3) while it was completely abolished in all mutants which were affected in Ser14 (compare lanes 6, 8, 9, and 10 to lane 1). Thus, only Ser14 and not Ser9 or Ser10 is phosphorylated in the rat α3 fusion proteins. This result is important since it implies that α3 isoforms of other species, which do not contain Ser14 (see Figure 5), are not substrates for PKC.

DISCUSSION

By a combination of site-directed mutagenesis and molecular modeling of the interaction of the α N-terminus with the active site of PKC, we characterized the basic structural requirements for PKC phosphorylation of the α1 isoform of Na,K-ATPase. Furthermore, phosphorylation studies on α2 and α3 isoforms revealed that, in contrast to α1 isoforms, these are poor substrates for PKC phosphorylation.

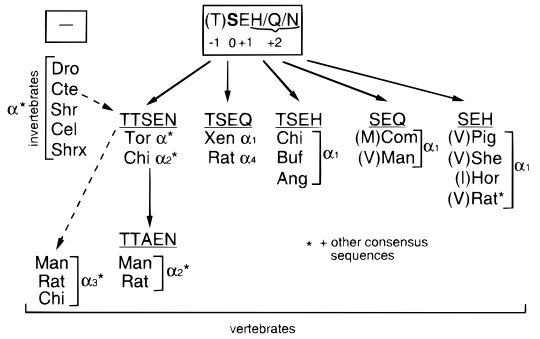

FIGURE 10: Phylogenetic aspects of the Ser-Glu-His motif. The figure shows the presence or absence of the Ser-Glu-His motif and its variants in the N-terminus of α subunits. An asterisk indicates the presence of consensus phosphorylation sites in the N-terminus. Amino acid residues preceding the Ser-Glu-Gln or the Ser-Glu-His motif are in brackets. For further explanations see text. Dro = *Drosphila*, Cte = *Ctenocephalides*, Shr = shrimp, Cel = *Hydra*, Chi = chicken, Tor = *Torpedo*, Xen = *Xenopus*, Buf = *Bufo*, Ang = *Anguilla*, Com = *Catostomus comersoni*, She = sheep, and Hor = horse.

A first result of the present study was the identification and characterization of a (Thr)-Ser-X-His motif which permits efficient PKC-mediated phosphorylation of α1 isoforms of Na,K-ATPase. PKC phosphorylation has been demonstrated in a great number of proteins and though no strict rules could be defined for a PKC phosphorylation motif, the phosphorylation actually occurs on serine and/or threonine residues contained in a sequence which is characterized by one or several positively charged arginine or lysine residues at position +1 through +3 and/or −1 through −3 relative to the phosphorylated serine or threonine residue (Kennely & Krebs, 1991). Synthetic peptides with the motif X-Arg-X-X-Ser-X-Arg-X tend to be the best substrates for PKC (Pearson & Kemp, 1991). In this study, we show that PKC motifs can deviate considerably from these predictions. In the N-termini of most α1 isoforms of Na,K-ATPase, a single histidine is present at position +2 relative to the phosphorylated serine residue which, in concert with more distantly located arginine and lysine residues, is necessary and sufficient for an efficient PKC phosphorylation. The strong electrostatic interaction of the histidine with PKC appears to be important since we observe a much lower phosphorylation of *Xenopus* and human α1 isoforms which possess a glutamine at position +2 as well as of *Bufo* α1 subunit mutants in which histidine was replaced by either glutamine or asparagine. Molecular modeling predicts, in addition, that the more distantly located positive residues which are particularly abundant in the N-terminus of α subunits, provide electrostatic interactions with negatively charged regions in the PKC and thus might be important for a correct positioning of the substrate in the active site of PKC.

It is interesting to follow the appearance of the (Thr)-Ser-Glu-His motif in the α1 subunits during Na,K-ATPase evolution. In identified α subunits of invertebrate no analogous sequences are present (see Figure 10), but instead they contain one or several consensus PKC sites in the

N-terminus. In vertebrates, we find the Ser-Glu sequence, which is maintained throughout evolution, in the most primitive, identified *Torpedo* α subunit. The strict conservation of the glutamate is interesting in view of our finding that it can be replaced by an alanine without a change in the phosphorylation of the *Bufo* α1 subunit. In the *Torpedo* α subunit, the amino acid at position +2 relative to the serine residue is an asparagine which permits only weak PKC-mediated phosphorylation. Finally, the *Torpedo* α subunit also shows for the first time in evolution a threonine at position −1 which accounts for about 10% of the PKC-mediated phosphorylation of the *Bufo* α1 subunit. It appears that the *Torpedo* α subunit containing a Thr-Thr-Ser-Glu-Asn motif occupies a key position in the evolution of the diversity of PKC motifs of α isoforms. From the evolutionary tree of the Na,K-ATPase family, deduced from the global sequence identity of the α subunits, it might be inferred that *Torpedo* α subunits are more ancient than the direct precursors of the α3 and α2 isoforms (Horisberger, 1994). Thus, perhaps in α3 isoforms, the Thr-Thr-Ser-Glu-Asn motif has been lost while other consensus PKC motifs were maintained. Furthermore, it might be speculated that the Thr-Thr-Ala-Glu-Asn sequence found in the α2 isoforms of higher vertebrates evolved from the Thr-Thr-Ser-Glu-Asn motif present in the *Torpedo* α as well as in the chicken α2 isoform. Finally, a Thr-Ser-Glu-His/Gln sequence is characteristic of α1 isoforms of lower vertebrate species (with the exception of *C. commersoni*) while in α1 isoforms of higher vertebrates the threonine residue is absent and the motif becomes Ser-Glu-His. An interesting and so far only known exception to this rule is the human α1 isoform which bears the Ser-Glu-Gln sequence.

An extension of our studies to α2 isoforms reveals that they are also phosphorylated in the N-terminus but, in contrast to α1 isoforms containing the Ser-X-His motif, they are poor substrates for PKC. The low phosphorylation observed in the rat and the chicken α2 is probably due to phosphorylation of the Thr-Thr-Ala-Glu-Asn or the Thr-Thr-Ser-Glu-Asn motif, respectively, and not of the more consensus PKC site coexisting in α2 isoforms (see Figure 5). The phosphorylated α2 sequence resembles the H18N mutant of the *Bufo* α1 subunit. Correspondingly, it results in a drastic reduction of the phosphorylation of all α2 isoforms compared to that of α1 isoforms. In α2 and in the H18N mutant, the remaining interactions with PKC are most likely due to the strong positive charges in positions 22−28 (see Figure 5) which may interact with the negative loops on PKC (see Figure 4). As to α3 isoforms, our data suggest that, with the exception of rat α3, they are not phosphorylated by PKC. Indeed, in rat α3, phosphorylation does not occur on the most N-terminal consensus PKC site which is conserved in all characterized α3 isoforms but instead in another rat-specific, consensus site (Lys-Lys-^{14}Ser-Lys) (see Figure 5). According to the sequence alignment, the phosphorylated Ser14 of the rat α3 corresponds to Ser23 in the rat α1 which is strongly phosphorylated. In both cases, a PKC consensus phosphorylation motif is present. However, a major difference between rat α1 and α3 resides in the presence of a proline in the −3 position in the rat α3. This proline residue might not only reduce the phosphorylation of Ser14 in rat α3 but also prevent the phosphorylation of Ser9/10 in all α2 and α3 sequences, probably due to an incompatibility of the local substrate structure.

The physiological significance of PKC phosphorylation of Na,K-ATPase in general, as well as of the regulatory implications of the variable phosphorylation of different α isoforms, remains to be established. The N-terminus is an interesting domain of the α subunits with respect to the possible functional consequences of PKC phosphorylation. It appears that it is directly involved in conformational changes of the Na,K-ATPase and in the associated occlusion and deocclusion of cations [for review and references see Horisberger (1994)]. It might be expected that an introduction of a negative phosphate group into the highly positively charged N-terminus could perturb its structural integrity and certain functional properties. However, so far, no studies exist which demonstrate a convincing correlation between PKC phosphorylation and a modulation of Na,K-ATPase activity.

We do not know the actual affinity for PKC of the native α isoforms but only their relative efficiency of phosphorylation. Indeed, K_m values cannot be determined in intact cells, and we deliberately renounced to perform such measurements on fusion proteins or peptides since they would be irrelevant for the native enzyme. Nevertheless, from our mutational analysis and molecular modeling, we may draw several conclusions on the potential physiological importance of PKC phosphorylation of Na,K-ATPase isozymes. In view of the high conservation of the Ser-Glu-His motif in the α1 isoform, the good molecular fit of the interaction of its N-terminus with PKC, and its relatively efficient phosphorylation, it can be predicted that the α1 isoform (perhaps with the exception of the human α1 subunit) is a possible target for regulatory phosphorylation by PKC in higher vertebrates. As to the α2 isoform, the putative consensus PKC site in the N-terminus is not phosphorylated, and it contains the phosphorylation motif lacking the serine and the histidine residues. As a consequence, α2 subunits are very poor substrates for PKC phosphorylation and probably also for PKC modulation. Species-specific adaptations exist, e.g., in chicken α2 isoforms which have maintained the serine residue and show a more efficient phosphorylation. Finally, α3 isoforms are the most unlikely candidates for regulatory phosphorylation. Indeed, with the exception of rat α3 which is phosphorylated to a low extent on a distinct consensus serine, they are not phosphorylated by PKC.

Thus, altogether, our results suggest that only α1 isoforms are possible targets for modulation by PKC phosphorylation. This conclusion is supported by the recent observation that in rat peripheral nerves, despite the presence of both α1 and α2 isoforms, phosphorylation by phorbol esters is associated only with α1 isoforms (Borghini et al., 1994) and apparently correlates with the effect of PKC modulators on the Na,K-pump activity in diabetic nerves [see Borghini et al. (1994) and references therein].

ACKNOWLEDGMENT

The cDNAs for rat α1, α2, and α3 isoforms and rat β1 were provided to us by J. Lingrel; the cDNA for human α1 was provided by K. Kawakami and the cDNA for chicken α2 by K. Takeyasu. We are thankful for these generous gifts. We also thank S. Cottecchia, J.-D. Horisberger, and Bernard Thorens for critical reading of the manuscript.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.

Beguin, P., Beggah, A. T., Chibalin, A. V., Burgenerkairuz, P., Jaisser, F., Mathews, P. M., Rossier, B. C., Cotecchia, S., & Geering, K. (1994) *J. Biol. Chem. 269*, 24437−24445.

Beguin, P., Beggah, A., Cotecchia, S., & Geering, K. (1996) *Am. J. Physiol. 270*, C131−C137.

Bertorello, A. M., & Katz, A. I. (1993) *Am. J. Physiol. 265*, F743-F755.

Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C., & Greengard, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11359−11362.

Borghini, I., Geering, K., Gjinovci, A., Wollheim, C. B., & Pralong, W. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6211−6215.

Boyle, W. J., Van der Geer, P., & Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.

Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., & Karplus, M. (1983) *J. Comput. Chem. 4*, 187−217.

Burgener-Kairuz, P., Horisberger, J. D., Geering, K., & Rossier, B. C. (1991) *FEBS Lett. 290*, 83−86.

Chibalin, A. V., Vasilets, L. A., Hennekes, H., Pralong, D., & Geering, K. (1992) *J. Biol. Chem. 267*, 22378−22384.

Feschenko, M. S., & Sweadner, K. J. (1994) *J. Biol. Chem. 269*, 30436−30444.

Feschenko, M. S., & Sweadner, K. J. (1995) *J. Biol. Chem. 270*, 14072−14077.

Fisone, G., Cheng, S. X. J., Nairn, A. C., Czernik, A. J., Hemmings, H. C., Hoog, J. O., Bertorello, A. M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., & Greengard, P. (1994) *J. Biol. Chem. 269*, 9368−9373.

Geering, K. (1995) in *Advances in Molecular & Cell Biology*, Life Science Programs, Greenwich, CT (in press).

Geering, K., Kraehenbuhl, J.-P., & Rossier, B. C. (1987) *J. Cell Biol. 105*, 2613−2619.

Geering, K., Theulaz, I., Verrey, F., Häuptle, M. T., & Rossier, B. C. (1989) *Am. J. Physiol. 257*, C851−C858.

Girardet, M., Geering, K., Frantes, J. M., Geser, D., Rossier, B. C., Kraehenbuhl, J.-P., & Bron, C. (1981) *Biochem. J. 20*, 6684−6691.

Good, P. J., Welch, R. C., Barkan, A., Somasekhar, M. B., & Mertz, J. E. (1988) *J. Virol. 62*, 944−953.

Greer, J. (1990) *Proteins: Struct., Funct., Genet. 7*, 317−334.

Horisberger, J.-D. (1994) *The Na,K-ATPase: Structure−Function Relationship*, pp 1−130, R. G. Landes Co., Austin, TX.

Huang, X., & Miller, M. (1991) *Adv. Appl. Math. 12*, 337−357.

Jaisser, F., Canessa, C. M., Horisberger, J. D., & Rossier, B. C. (1992) *J. Biol. Chem. 267*, 16895−16903.

Jaunin, P., Horisberger, J. D., Richter, K., Good, P. J., Rossier, B. C., & Geering, K. (1992) *J. Biol. Chem. 267*, 577−585.

Jørgensen, P. L., & Collins, J. H. (1986) *Biochim. Biophys. Acta 860*, 570−576.

Jørgensen, P. L., & Farley, R. A. (1988) *Methods Enzymol. 156*, 291−301.

Kawakami, K., Ohta, T., Nojima, H., & Nagano K. (1986) *J. Biochem. 100*, 389−397.

Kemp, B. E., Parker, M. W., Hu, S. H., Tiganis, T., & House, C. (1994) *Trends Biochem. Sci. 19*, 440−444.

Kennelly, P. J., & Krebs, E. G. (1991) *J. Biol. Chem. 266*, 15555−15558.

Lowndes, J. M., Hokinneaverson, M., & Bertics, P. J. (1990) *Biochim. Biophys. Acta 1052*, 143−151.

Luethy, R., Bowie, J. U., & Eisenberg, D. (1992) *Nature 356*, 83−85.

McDonough, A. A., & Farley, R. A. (1993) *Curr. Opin. Nephrol. Hypertens. 2*, 725−734.

McDonough, A. A., Wang, J. N., & Farley, R. A. (1995) *J. Mol. Cell. Cardiol. 27*, 1001−1009.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035−7056.

Middleton, J. P., Khan, W. A., Collinsworth, G., Hannun, Y. A., & Medford, R. M. (1993) *J. Biol. Chem. 268*, 15958−15964.

Nelson, R. M., & Long, G. L. (1989) *Anal. Biochem. 180*, 147−151.

Orr, J. W., & Newton, A. C. (1994) *J. Biol. Chem. 269*, 27715−27718.

Pearson, R. B., & Kemp, B. E. (1991) *Methods Enzymol. 200*, 62−81.

Peitsch, M. C. (1996) *Biochem. Soc. Trans. 24*, 274−279.

Ponder, J. W., & Richards, F. M. (1987) *J. Mol. Biol. 193*, 775−791.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Shull, G. E., Greeb, J., & Lingrel, J. B. (1986a) *Biochemistry 25*, 8125−8132.

Shull, G. E., Lane, L. K., & Lingrel, J. B. (1986b) *Nature 231*, 429−431.

Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31−40.

Takeyasu, K., Lemas, V., & Fambrough, D. (1990) *Am. J. Physiol. 259*, C619−C630.

Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., & Sowadski, J. M. (1992) *Annu. Rev. Cell Biol. 8*, 429−462.

Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., & Zoller, M. J. (1993) *Trends Biochem. Sci. 18*, 84−89.

Verrey, F., Kairouz, P., Schaerer, E., Fuentes, P., Geering, K., Rossier, B. C., & Kraehenbuhl, J. P. (1989) *Am. J. Physiol. 256*, F1034−F1043.

Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Xuong, N., Taylor, S. S., & Sowadski, J. M. (1993) *Biochemistry 32*, 2154−2161.

BI960516O