# Protein Kinase C Phosphorylation of Purified Na,K-ATPase: C-Terminal Phosphorylation Sites at the $\alpha$- and $\gamma$-Subunits Close to the Inner Face of the Plasma Membrane

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ABSTRACT The $\alpha$-subunit of the Na,K-ATPase is phosphorylated at specific sites by protein kinases A and C. Phosphorylation by protein kinase C (PKC) is restricted to the N terminus and takes place to a low stoichiometry, except in rat. Here we show that the $\alpha$-subunit of shark Na,K-ATPase can be phosphorylated by PKC at C-terminal sites to stoichiometric levels in the presence of detergents. Two novel phosphorylation sites are possible candidates for this PKC phosphorylation: Thr-938 in the M8/M9 loop located very close to the PKA site, and Ser-774, in the proximal part of the M5/M6 hairpin. Both sites are highly conserved in all known $\alpha$-subunits, indicating a physiological role. A similar pattern of detergent-mediated phosphorylation by PKC was found in pig kidney Na,K-ATPase $\alpha$-subunit. Interestingly, the kidney-specific $\gamma$-subunit was phosphorylated by PKC in the presence of detergent. The close proximity of the novel PKC sites to the membrane suggests that targeting proteins to tether PKC into the membrane phase is important in controlling the in vivo phosphorylation of this novel class of membrane-adjacent PKC sites. It is suggested that in purified preparations where functional targeting may be impaired detergents are needed to expose the sites.

## INTRODUCTION

The Na,K-ATPase is an integral membrane protein that transports $Na^+$ and $K^+$ across the plasma membrane of animal cells against their concentration gradients, using energy from the hydrolysis of ATP (Cornelius, 1996). The activity of Na,K-ATPase is essential for many cell functions and is strictly controlled by hormones, neurotransmitters, and growth factors (Ewart and Klip, 1995). The catalytic subunit of the Na,K-ATPase is a substrate for protein kinases and is phosphorylated both by cyclic AMP dependent-protein kinase (PKA), and $Ca^{2+}$/phospholipid-dependent protein kinase (PKC) (Beguin et al., 1994). This is believed to form the molecular basis for the rapid modulation of the Na,K-ATPase activity in response to hormonal stimulation (Cornelius et al., 2001). However, the effect of kinase phosphorylation on Na,K-ATPase activity in vivo remains a controversial matter and several seemingly inconsistent observations need further investigation (Féraille et al., 2000; Feschenko and Sweadner, 1997; Efendiev et al., 2000).

Two PKC-sites are located in the N-terminal part of the $\alpha$-subunit: one (Ser-18) is present only in the rat enzyme and is phosphorylated to stoichiometric levels, whereas another (Ser-11), is well conserved but is phosphorylated to very low levels (Feschenko and Sweadner, 1995). The PKA phosphorylation site (Ser-942) is conserved among all known $\alpha$-isoforms and is present in a small cytoplasmic loop between the M8/M9 transmembrane segments of the $\alpha$-subunit (Fisone et al., 1994; Feschenko and Sweadner, 1994). In purified Na,K-ATPase-containing membranes, phosphorylation by PKA requires the presence of detergents (Chibalin et al., 1992, 1993; Fisone et al., 1994; Feschenko and Sweadner, 1994), with Triton X-100 (TX-100) being the most effective. Studies in vivo have also indicated that activation of the PKA signaling pathway does not necessarily lead to phosphorylation of the Na,K-ATPase $\alpha$-subunit as recently discussed in detail (Feschenko et al., 2000). Such observations seem to question a physiological role of PKA phosphorylation at this site. Alternatively, the inaccessibility of Ser-942 in purified preparations may indicate that some essential component(s) or signaling events have been disrupted or lost during purification.

Several reports have indicated cross-talk between the PKA and PKC signaling pathways leading to phosphorylation of the Na,K-ATPase (Borghini et al., 1994; Cheng et al., 1997; Feschenko et al., 2000). This indication is surprising, because the conventional PKA- and PKC phosphorylation sites seem to be widely separated in the membrane, as inferred from the three-dimensional (3-D) structure of the closely related P-type ATPase, the sarcoplasmic reticulum Ca-ATPase (Toyoshima et al., 2000).

The present study was primarily initiated by the observation that the level of PKC-phosphorylation of the $\alpha$-subunit of Na,K-ATPase from shark rectal gland increased substantially in the presence of detergent. We report here the identification of previously unrecognized PKC sites in the C-terminal part of the $\alpha$-subunit of both shark rectal and pig renal Na,K-ATPase. $K^+$ ions significantly promote phosphorylation at these novel sites, suggesting a specific

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Unless otherwise indicated, the amino acid sequences in this study are numbered according to the primary sequence of the $\alpha$-subunit from *Torpedo californica* (Kawakami et al., 1985).



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interaction between PKC and the E2 conformation of the enzyme. Interestingly, the kidney-specific $\gamma$-subunit is also phosphorylated by PKC in the presence of detergent.

## METHODS AND MATERIALS

### Na,K-ATPase purification and solubilization

Purification of Na,K-ATPase-enriched membrane fragments was as previously described (Skou and Esmann, 1979). Protein concentration, ranging from 3–5 mg/ml, was determined using Peterson's modification of the Lowry method (Peterson, 1977), using bovine serum albumin as a standard. The ATPase activity was measured in a reaction mixture containing (in mM), 30 histidine, pH. 7.4, 130 NaCl, 20 KCl, 4 $MgCl_2$, 3 ATP ($Na^+$-salt). The concentration of $P_i$ hydrolyzed from ATP was measured as described by Baginski et al. (1967). The maximum specific activity was ~30 U/mg at 37°C and 10.5 U/mg at 24°C (1U = 1 $\mu$mole $P_i$/min). Partitioning of different concentrations of octa-ethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$) into the membrane-bound enzyme was performed by incubating the membrane-bound enzyme with the specified amount of detergent at 0°C for 10 min.

### Tryptic cleavage of the Na,K-ATPase $\alpha$-subunit

N-terminal truncation of the $\alpha$-subunit was performed by incubating membrane-bound enzyme with trypsin (trypsin to protein ratio of 1:100 (w/w)) for 10 min on ice in the presence of 100 mM NaCl and 1 mM EDTA, as previously described (Beguin et al., 1994). The truncation of the N terminus was confirmed by an increase in the mobility of the truncated $\alpha$-subunit in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For preparation of the so-called "19-kDa membranes," membrane-bound enzyme was incubated with trypsin (trypsin to protein ratio of 1:5 (w/w)) for 1 h at 37°C, in the presence of 20 mM KCl and 1 mM EDTA, as previously described (Karlish et al., 1991). In both cases the reaction was started by the addition of trypsin and stopped by addition of a 10-fold excess of soybean trypsin inhibitor. The mixtures were diluted 10-fold with ice cold imidazole buffer (25 mM), and centrifuged at 170,000 *g* for 1 h at 10°C. The membranes were washed with imidazole buffer and centrifuged again, then finally suspended in 30 mM histidine, pH 7.4, containing 25% glycerol and stored at −20°C.

Preparation of 19-kDa membranes lacking the M5/M6 hairpin was essentially as previously described (Lutsenko et al., 1995). Briefly, post-tryptic membrane-bound Na,K-ATPase was incubated in 25 mM imidazole, 1 mM EDTA, and 20 mM Tris, pH 7.4 (to obtain release of the M5/M6 fragment) or in buffer where $K^+$ replaced Tris (to obtain intact 19-kDa membranes) for 10 min at 37°C. The mixtures were centrifuged as described above and the membranes were stored at −20°C in the same buffers.

To measure the phosphorylation intensity as a function of the $K^+$ concentration, freshly prepared 19-kDa membranes were washed twice in $K^+$-free imidazole buffer and phosphorylated at increasing $K^+$ concentrations.

### PKA and PKC phosphorylation of the Na,K-ATPase

PKA phosphorylation was performed as previously described (Cornelius and Logvinenko, 1996) in a reaction mixture containing 50 mM Hepes, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM ATP (Tris-salt), 4 $\mu$g protein, a detergent concentration as indicated in legends to figures, and 3 U of PKA. The catalytic subunit of PKA was purchased from Sigma (St. Louis, MO). PKC phosphorylation was performed in a typical assay mixture containing: 50 mM Hepes, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.02 mM L-$\alpha$ phosphatidylserine (Avanti Polar Lipids, Alabaster, AL), 0.01 mM dioleoyl 1,2-*sn*-glycerol (Sigma), 0.1 mM ATP (Tris salt), 4 $\mu$g protein, and 0.13 $\mu$g of PKC. PKC phosphorylation in mixed micelle assay contained the same ligands plus detergent concentrations as indicated in legends to figures. PKC was from CalBiochem (La Jolla, CA) and contained the $Ca^{2+}$-dependent isoforms. The phosphorylation reaction for both kinases was initiated by the addition of ATP (containing 3 $\mu$Ci/pmol [$^{32}$P]ATP), allowed to proceed for 30 min at 24°C, and terminated by the addition of 16-$\mu$l sample buffer (Laemmli, 1970). For PKC phosphorylation before fingerprinting (see below), $Ca^{2+}$ ions were removed by the inclusion of EDTA in the trypsinization buffer. Calculation of phosphorylation stoichiometry was performed using a measured phosphoenzyme (EP)-level of 2.5 nmol/mg protein as previously described (Cornelius and Logvinenko, 1996).

### Gel electrophoresis and immunoblotting

The phosphorylated proteins were separated using SDS-PAGE (3% stacking gel, 9% intermediate, and 16% resolving gels, unless otherwise indicated). The gels were stained with Coomassie blue, destained, and dried, then analyzed by autoradiography overnight at −80°C. The phosphorylated bands corresponding to the $\alpha$-subunit were excised from the gels and the radioactivity measured in a scintillation counter. The phosphorylation stoichiometry was calculated from the radioactivity associated with the $\alpha$-subunits, the amount of the protein in the preparation, and the purity determined from the phosphorylation level, as previously described (Cornelius and Logvinenko, 1996). For immunoblotting after electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, Hercules, CA), then washed three times for 20 min with phosphate-buffered saline (PBS) and incubated over night at room temperature with the primary antibody, as described in results and in legends to figures. The PVDF membranes were washed again with PBS and incubated with goat anti-rabbit antibody for 2 h. After washing, the proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia, Peapack, NJ ). Detection of phosphorylation at the C-terminal threonine residue of the Na,K-ATPase $\alpha$-subunit was performed using a specific anti-phosphothreonine antibody (dilution 1:200; Chemicon International, Temecula, CA). Anti-phosphoserine antibody, used at the same dilution, was used as a control to distinguish between serine and threonine phosphorylation. For parallel detection of threonine and serine phosphorylation, all incubations and exposure times were identical in the two cases to allow proper comparison between phosphorylation at threonine and serine residues, respectively.

### Proteolytic fingerprinting

These experiments were essentially performed as previously described (Sweadner, 1991). PKC phosphorylation products containing electrophoresis sample buffer were treated with 5 $\mu$g of soybean trypsin inhibitor (in 150 mM NaCl, 5 mM EDTA). Shortly before loading to gels, 0.8 $\mu$g trypsin is added (trypsin to protein ratio of 1:5 (w/w)), and volumes containing 2 $\mu$g protein were loaded onto 14% Tricine gels. In control samples, the same volume of buffer without trypsin was added. Electrophoresis was run for 10–16 h then the samples were transferred to PVDF membranes and stained with Coomassie blue or washed with PBS for subsequent antibody staining, as described above. The C-terminal fragments of the $\alpha$ subunit were detected using the C-terminal specific antibody NKA1002–16 (antibody raised against the sequence 1002–1016 of the pig $\alpha$-subunit, kindly provided by Dr. J.V. Møller). Phosphorylation of proteins (or protein fragments) was measured by autoradiography.

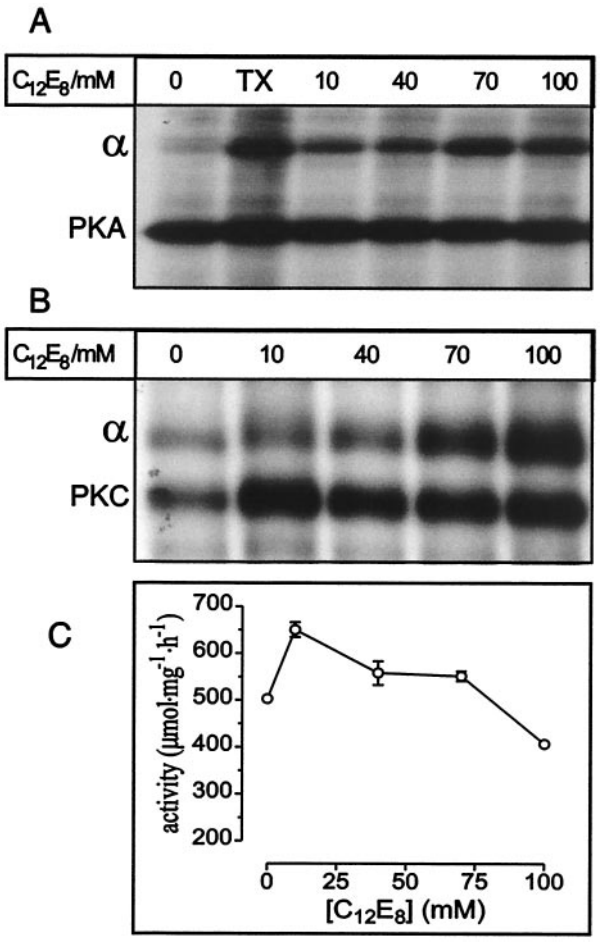



FIGURE 1 Detergent-mediated protein kinase phosphorylation of Na,K-ATPase $\alpha$-subunit. (*A*) Autoradiogram showing PKA phosphorylation of membrane-bound shark Na,K-ATPase in the absence of detergent (lane 1), in the presence of 1.6 mM TX-100 (lane 2), or in the presence of increasing concentrations of $C_{12}E_8$ (lanes 3–6 indicate $C_{12}E_8$ concentrations of 10, 40, 70, and 100 mM, respectively). The membranes were pre-incubated with $C_{12}E_8$ for 10 min on ice and 2 $\mu$l aliquots were added to the PKA phosphorylation mixture (50 $\mu$l), as described in Materials and Methods. The phosphorylation products were separated by SDS-PAGE followed by visualization of phosphorylation by autoradiography. After autoradiography of the dry gels, the bands corresponding to the $\alpha$-subunits were excised from the gel and the radioactivity measured in a scintillation counter. The phosphorylation stoichiometry (mean $\pm$ SE) of the $\alpha$-subunit in the different lanes (in mol $P_i$/mol $\alpha$-subunit) was calculated from the protein content added to each lane and the measured EP-level of 2.5 nmol/mg as previously described (Cornelius and Logvinenko, 1996) and is as follows: 0.007 $\pm$ 0.002 (lane 1), 0.81 $\pm$ 0.06 (lane 2), 0.21 $\pm$ 0.01 (lane 3), 0.31 $\pm$ 0.05 (lane 4), 0.59 $\pm$ 0.07 (lane 5), and 0.76 $\pm$ 0.06 (lane 6). Results from two independent experiments are shown. (*B*) Autoradiogram showing PKC phosphorylation of membrane-bound shark Na,K-ATPase (labeled 0 mM $C_{12}E_8$), or of enzyme in the presence of increasing $C_{12}E_8$ concentrations, as indicated at the top of the figure. The membranes were pre-incubated with $C_{12}E_8$ and 2 $\mu$l aliquots were added to the PKC phosphorylation mixture (50 $\mu$l), as described in Materials and Methods. The phosphorylation stoichiometry (mean $\pm$ SE) calculated as described above was 0.050 $\pm$ 0.015 (lane 1), 0.113 $\pm$ 0.04 (lane 2), 0.191 $\pm$ 0.005 (lane 3), 0.292 $\pm$ 0.002 (lane 4), and 0.750 $\pm$ 0.03 (lane 5). The result is representative of seven independent measurements. (*C*) Maximum hydrolytic activity at 23°C as a function of the $C_{12}E_8$ concentration. Enzyme was pre-equilibrated with the specified $C_{12}E_8$ concentration and the maximal hydrolytic activity measured as described in Materials and Methods. $C_{12}E_8$ increased the enzyme activity at all concentrations up to 100 mM, where a slight decrease in activity to ~75% of control is observed. Points are mean of three determinations $\pm$ SE.

## RESULTS

### TX-100- and $C_{12}E_8$-mediated PKA phosphorylation of shark rectal Na,K-ATPase

Fig. 1 *A* shows an autoradiogram of a typical experiment comparing PKA phosphorylation of membrane-bound shark rectal Na,K-ATPase before (lane 1) and after addition of detergents such as TX-100 (lane 2) and $C_{12}E_8$ (lanes 3–6). In the absence of detergents, no phosphorylation by PKA can be observed (lane 1), whereas stoichiometric phosphorylation occurs in the presence of 0.1% (1.6 mM, >5 $\times$ critical micelle concentration) TX-100 (lane 2). TX-100 seems to have specific effects other than to induce solubilization of the membrane, because 10 mM $C_{12}E_8$, a concentration routinely used in the solubilization of shark Na,K-ATPase membranes without loss of activity (Esmann, 1983; Esmann and Skou, 1984), was found insufficient to support optimal PKA phosphorylation of the $\alpha$-subunit. Increasing the $C_{12}E_8$ concentration resulted in an increase in the phosphorylation stoichiometry (Fig. 1 *A*, lanes 3–6), and saturated at ~100 mM, where the maximum phosphorylation level obtained became equivalent to the level obtained by 0.1% TX-100.

### PKC-mediated phosphorylation of the shark enzyme at the C terminus in the presence of detergent

PKC phosphorylation of several Na,K-ATPase $\alpha$-isoforms in purified membranes has been previously shown to occur to a low stoichiometry (Feschenko and Sweadner, 1995) because of the lack of the main PKC phosphorylation site Ser-18, which is present only in rat $\alpha$1 and $\alpha$3 isoforms. This was previously found to be the case also for the shark enzyme (Cornelius et al., 2000; Mahmmoud et al., 2000). As with PKA phosphorylation, a significant increase in the phosphorylation level could be demonstrated in the presence of increasing concentrations of $C_{12}E_8$ (up to 100 mM), as shown in Fig. 1 *B*. The increased phosphorylation intensity of the $\alpha$-subunit is probably not resulting from an increase in the PKC activity, as judged by the constant level of PKC autophosphorylation observed at increasing detergent concentrations.

As demonstrated in Fig. 1 *C*, the maximum hydrolytic activity increased at detergent concentrations as high as 75 mM and even at 100 mM $C_{12}E_8$, ~75% of the hydrolytic activity is retained.

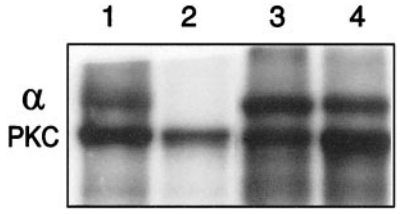



FIGURE 2 Detergent-induced PKC phosphorylation at C-terminal sites. Autoradiogram showing PKC phosphorylation of membrane-bound Na,K-ATPase from shark rectal glands at the following conditions: lane 1, membrane-bound Na,K-ATPase; lane 2, membrane-bound Na,K-ATPase in which the N-terminal segment is cleaved by controlled trypsin treatment (N-terminal truncated enzyme, see Material and Methods); lane 3, N-terminal truncated enzyme in the presence of 1.6 mM TX-100; and lane 4, N-terminal truncated enzyme in the presence of 50 mM $C_{12}E_8$. The phosphorylated proteins were separated on gradient SDS gel as described in Materials and Methods. Representative of two independent experiments is shown.

Truncation of the first 30 N-terminal residues of the Na,K-ATPase $\alpha$-subunit was previously shown to abolish PKC-phosphorylation of enzyme from duck salt glands, rabbit and sheep kidney (Beguin et al., 1994), and rat kidney (Feschenko and Sweadner, 1995), demonstrating that phosphorylation of the Na,K-ATPase $\alpha$-subunit by PKC in the absence of detergent is restricted to the N-terminal part. Whether phosphorylation of the shark $\alpha$-subunit in the presence of $C_{12}E_8$ occurred at the N terminus, or at alternative site(s) was investigated by comparing PKC-phosphorylation of native and N-terminal truncated enzyme in the presence or absence of detergent. As seen in Fig. 2, PKC-phosphorylation is evident in control enzyme in the absence of detergent (Fig. 2, lane 1), but absent in N-terminal truncated enzyme (lane 2), demonstrating that PKC-mediated phosphorylation of native shark enzyme is restricted to the N-terminal part. However, when N-terminal truncated enzyme was phosphorylated by PKC in the presence of TX-100 or $C_{12}E_8$, phosphorylation became evident (lanes 3 and 4, respectively), indicating that the detergent treatment exposed sites additional to the N-terminal ones. Moreover, the intensity of the PKC-phosphorylation of the N-terminal truncated enzyme seemed to be higher in the presence of detergent than for PKC-phosphorylation to the N-terminal sites in the absence of detergent.

The location of the PKC-phosphorylation sites that are exposed by detergent was further characterized by proteolytic fingerprinting (Sweadner, 1991). The standard mapping of tryptic fragments in the presence of $Na^+$ or $K^+$ ions (Jørgensen and Collins, 1986; Jørgensen and Farley, 1988) can not be applied here because of the anomalous tryptic pattern found in the presence of $C_{12}E_8$ and SDS, as previously reported (Fotis et al., 1999). Initially, it was determined whether or not the small N terminus of the $\alpha$-subunit is tryptically cleaved off in the presence of detergent. If the first 30 N-terminal amino-acids fragment of the $\alpha$-subunit is split off by trypsin, it will not be resolved in a 12–14% SDS/Tricine-gel. However, if the cleavage of the small N terminus is protected by detergent and therefore still asso-

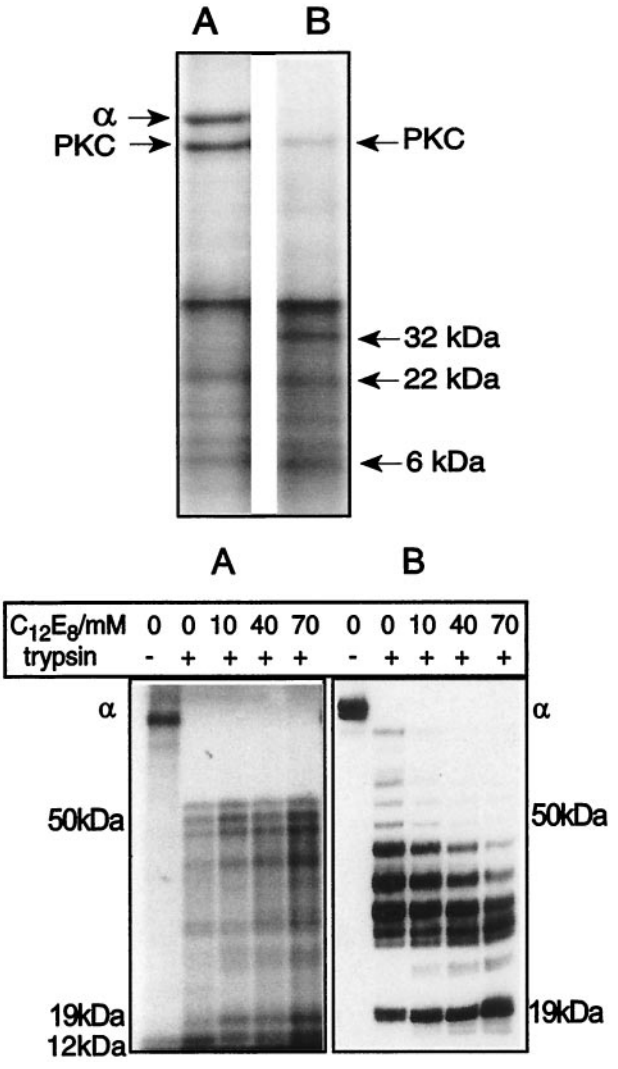



FIGURE 3 Proteolytic fingerprinting of PKC-phosphorylated membrane-bound and solubilized shark Na,K-ATPase. *Upper panel* shows PKC phosphorylation in the absence of detergent of shark Na,K-ATPase before (*A*) and after proteolytic fingerprinting (*B*), demonstrating that phosphorylation is associated with a 32-kDa fragment. *Lower panel* shows an autoradiogram of PKC phosphorylated shark Na,K-ATPase after proteolytic fingerprinting (*A*). Proteolytic fingerprinting was performed after phosphorylation by PKC of membrane-bound Na,K-ATPase (0 mM $C_{12}E_8$) or Na,K-ATPase solubilized by different $C_{12}E_8$ concentrations as indicated at the top of the figure. (*B*) shows an immunoblot of membrane-bound and solubilized shark enzyme in control (−trypsin) or after trypsin treatment (+trypsin). The antibody used was a C-terminal specific antibody raised against the peptide 1002–1016 of the $\alpha$-subunit. The panel shows that trypsinization of the enzyme in the presence of increasing detergent concentrations leads to an anomalous digestion pattern distinct from the standard pattern observed in the presence of $Na^+$ (Jørgensen and Collins, 1986). Reaction conditions were as outlined in Materials and Methods. Note that a 12-kDa phosphorylated fragment at the bottom of the gel (*A, lower panel*) which is not probed by the C-terminal specific antibody (*B*). The result is representative of four independent measurements.

ciated with a larger fragment of the $\alpha$-subunit, it will be resolved in the gel. As seen from Fig. 3, upper panel, proteolytic fingerprinting of shark $\alpha$-subunit after phosphorylation by PKC at the conventional N-terminal sites in the absence of detergent resulted in one major phosphorylated band. The band migrated at ~32 kDa in the gel (*B*), probably resulting from cleavage at the T3 position and not at the T2 position (Jørgensen and Collins, 1986). This demonstrates that the N terminus is protected against trypsinization in the presence of detergents. The 32-kDa fragment is absent in autoradiograms after phosphorylation and fingerprinting of N-terminal truncated enzyme (not shown). Thus, phosphorylation at this 32-kDa fragment after fingerprinting represents N-terminal phosphorylation.

As seen from Fig. 3, lower panel, proteolytic fingerprinting of the shark enzyme reveals the presence of several fragments that are phosphorylated by PKC (*A*). Consistent with the results shown in Fig. 1 *B*, phosphorylation of the trypsinized fragments increased proportionally with increasing $C_{12}E_8$ concentrations. Probing the phosphorylated fragments with a C-terminal specific-antibody (NKA 1002–16) demonstrated PKC phosphorylation at several C-terminal fragments of the $\alpha$-subunit (Fig. 3 *B*, *lower panel*), including the 19-kDa fragment that comprises the segment from an asparagine residue at the transmembrane domain M7 to the C terminus at Tyr-1022.

It is possible that other sites located in the middle part of the $\alpha$-subunit are also exposed by detergent and phosphorylated by PKC in the intact $\alpha$-subunit. This possibility is supported by the observation that detergent-mediated phosphorylation is also detected at a 12-kDa fragment (Fig. 3 *A*, *lower panel*), which is not probed with NKA1002–16 (Fig. 3 *B*, *lower panel*), indicating that it is not a C-terminal product of a further trypsinization of the 19-kDa fragments. It is also seen from Fig. 3, lower panel, that higher concentrations of $C_{12}E_8$ resulted in an increased amount of the 19-kDa fragment (Fig 3 *B*) indicating that high concentrations of $C_{12}E_8$ have a $K^+$-like effects on the conformation of the $\alpha$-subunit (see below).

That the detergent-induced PKC-phosphorylation is occurring at the C-terminal 19-kDa fragment was also demonstrated using isolated "19-kDa membranes" (Karlish et al., 1991). The so-called 19-kDa membrane preparation contains the C-terminal 19-kDa fragment and several smaller peptides of molecular mass 8–12 kDa representing pairs of transmembrane segments (M1/M2, M3/M4, and M5/M6) (Capasso et al., 1992; Shainskaya and Karlish, 1994). Fig. 4 *A* shows PKC-phosphorylation of the 19-kDa membranes from shark $\alpha$-subunit in the absence (*left panel*) or presence (*right panel*) of 0.1% of TX-100. As indicated, the 19-kDa fragment is clearly phosphorylated only in the presence of detergent. PKA phosphorylation of the 19-kDa fragment, which contains the PKA phosphorylation site, Ser-942, is also dependent on the presence of TX-100, as shown in Fig. 4 *B*. The PKC phosphorylation intensity of the 19-kDa fragment was lower than found for both the intact $\alpha$-subunit and the N-terminal truncated $\alpha$-subunit, indicating that other sites in the middle part of the protein may be exposed to detergent as well.

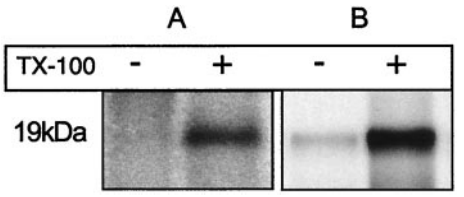


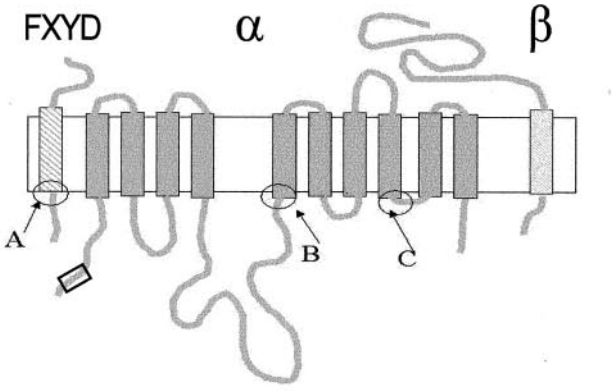

FIGURE 4 PKC and PKA phosphorylation of the 19-kDa membrane. Autoradiogram showing PKC (*A*) and PKA (*B*) phosphorylation of the 19-kDa membranes in the absence (*left lane*) or in the presence of 0.1% TX-100 (*right lane*). As indicated, phosphorylation of the 19-kDa fragment is only evident in the presence of detergent. Samples were resolved by gradient SDS gels as described in Materials and Methods. Phosphorylation by PKC of the 19-kDa fragment corresponded to ~30% of the phosphorylation level of N-terminal truncated enzyme, indicating that sites outside the 19-kDa fragment are phosphorylated and contribute to the high stoichiometry measured in Figs. 1 *B* and 2.

## PKC-phosphorylation in the M8/M9 cytoplasmic loop

The primary sequence of the C-terminal 19-kDa fragment of the $\alpha$-subunit shows considerable homology among different species and isoforms of the $\alpha$-subunit (Fig. 5), in contrast to the N-terminal fragment (Sweadner 1989; Blanco and Mercer, 1999). Screening of the C-terminal part of the $\alpha$-subunit for PKC-phosphorylation consensus motifs resulted in the identification of only one: in the sequence of *Torpedo californica* (Kawakami et al., 1985), an elasmobranch like the shark, Thr-938 (Thr-934 in the rat $\alpha$1) represents a consensus motif for PKC-phosphorylation (KTRR, Fig. 5 *C*). It is located in close proximity to the PKA phosphorylation site and is conserved in all known $\alpha$-isoforms. The presence of this putative PKC site at the M8/M9 loop only four amino acids upstream the PKA site is consistent with the detergent requirement for phosphorylation of both PKA and PKC at this loop.

## Detection of PKC phosphorylation of the $\alpha$-subunit using anti-phosphothreonine and anti-phosphoserine specific antibodies

Immunogenic-dependent methods have been used successfully for the detection of kinase phosphorylation at specific sites in the $\alpha$-subunit (Feschenko and Sweadner, 1997; Feschenko et al., 2000). In the present study we used two commercially available antibodies to detect phosphorylation at serine or threonine residues in shark rectal gland membrane proteins. To demonstrate the presence of a phosphorylated threonine residue at the $\alpha$-subunit C terminus immu-




A

| | | |
|---|---|---|
| FXYD2 Human | GLLILL | SRRFRCGG |
| FXYD2 Rat | GLLILL | SKRFRCGG |
| FXYD2 mouse | GLLIIL | SKRFRCGG |
| FXYD2 sheep | GLVIIL | SKRFRCGA |
| FXYD2 bovine | GLVIIL | SKRFRCGA |
| FXYD2 Xenopus | GMLLIF | SGRFRCGR |
| FXYD2 Pig | GLLILL | SKRFRCGG |
| FXYD1 Human | GILIVL | SRRCRCKF |
| FXYD1 Rat | GILLIL | SKRCRCKF |
| FXYD1 Dog | GILIVL | SRRCRCKF |
| FXYD3 Human | GIIIVM | SAKCKCKG |
| FXYD3 Pig | GIIILL | SGKCKCKF |

B

| | |
|---|---|
| *B. marinus* | VEEGRLIFDNLKK**S**$^{775}$IAYTLTSNIP |
| Catostomus | VEEGRLIFDNLKK**S**$^{779}$IAYTLTSNIP |
| Chicken | VEEGRLIFDNLKK**S**$^{773}$IAYTLTSNIP |
| Dog | VEEGRLIFDNLKK**S**$^{773}$IAYTLTSNIP |
| Drosophila | VEEGRLIFDNLKK**S**$^{790}$IAYTLTSNIP |
| Human $\alpha_2$ | VEEGRLIFDNLKK**S**$^{772}$IAYTLTSNIP |
| Human $\alpha_3$ | VEEGRLIFDNLKK**S**$^{765}$IAYTLTSNIP |
| Rat $\alpha_1/\alpha_2^{772}/\alpha_3^{765}$ | VEEGRLIFDNLKK**S**$^{775}$IAYTLTSNIP |
| Tilapia $\alpha_3$ | VEEGRLIFDNLKK**S**$^{762}$IAYTLTSNIP |
| Pig | VEEGRLIFDNLKK**S**$^{775}$IAYTLTSNIP |
| *T. Californica* | VEEGRLIFDNLKK**S**$^{774}$IAYTLTSNIP |

C

| | |
|---|---|
| Human α1 | V I C K T$^{934}$ R R N **S**$^{938}$ V F Q Q |
| α2 | I I C K T$^{931}$ R R N **S**$^{935}$ V F Q Q |
| α3 | I I C K T$^{924}$ R R N **S**$^{928}$ V F Q Q |
| α4 | I I C K T$^{939}$ R R N **S**$^{943}$ L F K Q |
| Rat α1 | V I C K T$^{934}$ R R N **S**$^{938}$ V F Q Q |
| α2 | I I C K T$^{931}$ R R N **S**$^{935}$ V F Q Q |
| α3 | I I C K T$^{924}$ R R N **S**$^{928}$ V F Q Q |
| Dog α1 | V I C K T$^{932}$ R R N **S**$^{936}$ V F Q Q |
| Pig α1 | V I C K T$^{932}$ R R N **S**$^{936}$ V F Q Q |
| *T. Californica* α1 | I I C K T$^{933}$ R R N **S**$^{937}$ I F Q Q |
| Sheep α1 | V I C K T$^{932}$ R R N **S**$^{936}$ V F Q Q |
| Tilapia α1 | I I C K T$^{921}$ R R N **S**$^{925}$ V F Q Q |
| Tilapia α3 | I I C K T$^{934}$ R R N **S**$^{938}$ V F Q Q |
| *Xenopus* α | I I C K T$^{936}$ R R N **S**$^{940}$ V F Q Q |

FIGURE 5 PKC phosphorylation of sites close to the inner face of the plasma membrane. Sketch of Na,K-ATPase subunits showing the location of the conventional N-terminal PKC-sites (*rectangle*) and the novel C-terminal consensus motifs at the M5/M6 and M8/M9 fragments of the α-subunit (*circles B and C*) together with the γ-subunit PKC-site (*circle A*). (*A*) PKC consensus site of the γ-subunit. (*B*) PKC consensus site in the M5/M6 hairpin. (*C*) PKA and PKC consensus sites in the sequence of the cytoplasmic loop between the transmembrane segment M8 and M9 (Kawakami et al., 1985). In all sequences the protein kinase site is indicated in bold. Some of the sequences were obtained from the GenBank (http://www.ncbi.nlm.nih.gov).

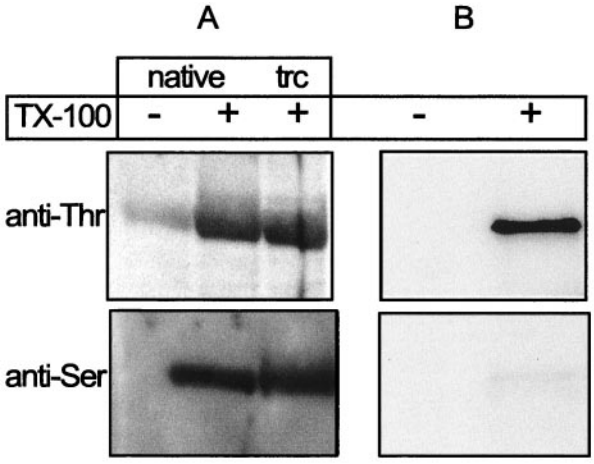



FIGURE 6 PKC phosphorylation of the α-subunit and the 19-kDa fragment probed by anti-phosphothreonine and anti-phosphoserine antibodies. Immunoblots showing PKC phosphorylation of membrane-bound or N-terminal truncated shark α-subunit (*A*) and the 19-kDa membranes (*B*) in the absence or presence of 0.1% TX-100 as indicated at the top of the figure. After phosphorylation proteins were electrotransferred to PVDF membranes and probed with an anti-phosphothreonine antibody (*top panels*) or an anti-phosphoserine antibody (*bottom panels*). Phosphorylated bands were visualized using the enhanced chemiluminescence reagents as described in Materials and Methods. Representative of two independent experiments is shown.

noblots after PKC phosphorylation of native membranes (controls), TX-100 solubilized α-subunit, and TX-100 solubilized N-terminal truncated α-subunit were compared using the two antibodies. The phosphorylation products were analyzed by SDS-PAGE, electrotransferred to PVDF membranes, and immunoblotted with anti-phosphothreonine (Fig. 6 *A*, *top panels*) or anti-phosphoserine (Fig. 6 *A*, *bottom panels*) antibodies. As seen, neither antibody detected phosphorylation after PKC phosphorylation of native membrane-bound enzyme (Fig. 6 *A*, *left lanes*), whereas with 0.1% TX-100 present in the PKC mixture (Fig. 6 *A*, *middle lanes*) both the anti-phosphothreonine and anti-phosphoserine antibody reacted. Furthermore, both antibodies probed phosphorylation by PKC of N-terminal truncated enzyme (labeled trc) in the presence of TX-100 (Fig. 6 *A*, right lanes). Finally, detergent-mediated PKC phosphorylation of the 19-kDa fragment could be demonstrated by the anti-phosphothreonine antibody (Fig. 6 *B*, *top panel*), but

not by the anti-phosphoserine antibody (Fig. 6 *B*, *bottom panel*), lending further evidence to the presence of a threonine in the 19-kDa fragment as the phosphorylation site for PKC.

## Detergent-mediated PKC phosphorylation of the M5/M6 hairpin

As seen from Fig. 6 *A*, detergent-supported phosphorylation of the $\alpha$-subunit by PKC could be detected using the anti-phosphoserine antibody (Fig. 6 *A*, *bottom panel*), both in the intact $\alpha$-subunit (*middle lane*) and after truncation of the N terminus (*right lane*). This indicates the presence of a phosphorylated serine residue outside the N terminus, probably located in the middle part of the $\alpha$-subunit (between amino acids Asp-31 and Asn-838, in the rat $\alpha$1 sequence). Because phosphorylation of this putative serine residue by PKC is observed only in the presence of detergents, it is likely that it is located in close proximity to the membrane. It is therefore conceivable that this site is located in one of the transmembrane hairpins M1/M2, M3/M4, or M5/M6. This is further supported by the proteolytic fingerprinting (Fig. 3 *A*, *lower panel*), in which a phosphorylated fragment is observed at 12 kDa that is not probed by the C-terminal antibody (Fig. 3 *B*, *lower panel*). Actually, a highly conserved PKC motif is present in the M5/M6 hairpin ($NLKKS^{774}$) located in the cytoplasm close to the membrane phase (Fig. 5 *B*). Therefore, it was investigated whether PKC phosphorylation at this site depended on the presence of TX-100 using a posttryptic preparation of the shark Na,K-ATPase.

In 19-kDa membranes of Na,K-ATPase (Lutsenko et al., 1995) and in H,K-ATPase (Gatto et al., 1999) prepared in the presence of KCl with a high trypsin to protein ratio, it has previously been shown that the M5/M6 hairpin is preferentially lost from the 19-kDa membranes after a brief incubation at 37°C in the absence of $K^+$ ions. Accordingly, 19-kDa membrane preparations thoroughly washed to remove $K^+$ ions were divided into two batches, one of which was incubated in imidazole buffer in the absence of $K^+$ (19-kDa membranes minus M5/M6), whereas the other was incubated in the same buffer containing 20 mM $K^+$ (19-kDa membrane plus M5/M6). After centrifugation, the pellets were suspended in the same buffer and both preparations phosphorylated by PKC in the absence, or in the presence of 1.6 mM TX-100. Figure 7 shows the results of phosphorylation by PKC of the two 19-kDa membrane preparations in the absence or presence of TX-100. Consistent with the results of Figs. 4 and 6, no phosphorylation was observed in the absence of detergent. However, in the presence of detergent, incubation of the intact 19-kDa membranes (labeled M5/M6+) with PKC resulted in the phosphorylation of both the 19-kDa fragment and a 12-kDa fragment, probably representing the M5/M6 hairpin. In the 19-kDa membrane lacking the M5/M6 hairpin (labeled M5/M6−) the 19-kDa fragment is phosphorylated by PKC to a lesser extent, and little or no phosphorylation of the 12-kDa fragment could be observed. This could indicate that the M5/M6 loop is required for PKC phosphorylation of both C-terminal sites. Together, these results indicate that the M5/M6 hairpin contains a serine residue that can be phosphorylated by PKC only in the presence of detergent.

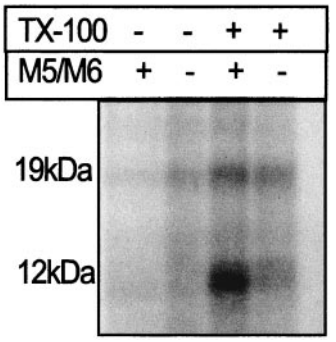



FIGURE 7 Detergent-mediated PKC phosphorylation of a site located in the M5/M6 hairpin of the shark $\alpha$-subunit. An autoradiogram showing PKC phosphorylation of the 19-kDa membranes incubated in a Tris-buffer (to obtain release of the M5/M6 fragment, labeled M5/M6−) or in a $K^+$-buffer (labeled M5/M6+), as indicated at the top of the figure. Phosphorylation of both the M5/M6 fragment and the M8/M9 fragment was dependent on the presence of TX-100. The 19-kDa fragment was phosphorylated whether or not the M5/M6 hairpin was present in the membranes, although to a lesser extent in the absence of the M5/M6 fragment. Representative of three independent experiments is shown.

The PKC phosphorylation intensity of the 19-kDa and the 12-kDa fragment, as well as of intact and truncated $\alpha$ was measured by autoradiography using the same amount of protein (2 $\mu$g) processed at identical conditions. The results are shown in Table 1. The phosphorylation intensity of the 19-kDa fragment amounts to ~33% of the truncated enzyme.

PKC phosphorylation of the sites located at the 19-kDa and 12-kDa fragments was stimulated by $K^+$ ions present in

**TABLE 1 PKC-mediated $P_i$ incorporation into intact and trypsin treated Na,K-ATPase**

| Fragment | $P_i$ incorporation | |
|---|---|---|
| | pmol/$\mu$g | mol $P_i$/mol $\alpha$ |
| Intact $\alpha$ | 0.83 ± 0.05 | 0.33 ± 0.02 |
| N-terminal truncated $\alpha$: | 2.71 ± 0.03 | 1.08 ± 0.01 |
| 19-kDa fragment | 0.90 ± 0.09 | 0.36 ± 0.04 |
| 12-kDa fragment | 1.68 ± 0.02 | 0.67 ± 0.01 |

The amount of PKC-mediated phosphate incorporated at the different shark $\alpha$-fragments. Intact $\alpha$ indicates phosphorylation at the N terminus (in the absence of detergent). Phosphorylation of N-terminal truncated $\alpha$ in the presence of TX-100 represents C-terminal phosphorylation (at both the 19- and 12-kDa fragments). Phosphate incorporation into either the 19-kDa or the 12-kDa fragments was also determined by PKC phosphorylation of the isolated 19-kDa membranes in the presence of TX-100 followed by SDS-PAGE to separate the fragments. Numbers are means ± SE.

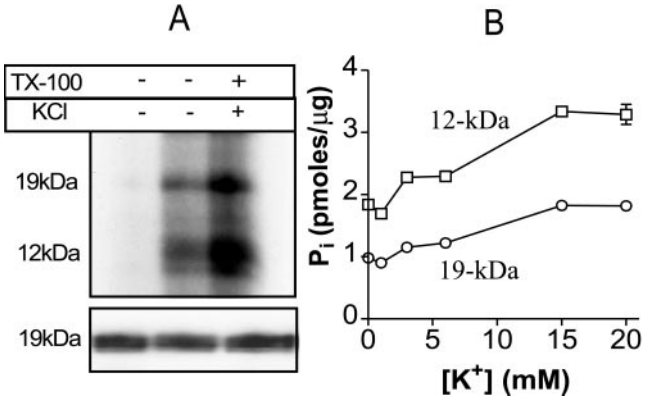



FIGURE 8 $K^+$ activation of PKC phosphorylation of the 12-kDa and 19-kDa fragments. (*A, upper frame*) Autoradiogram demonstrating PKC phosphorylation of the 19-kDa membrane in the absence or presence 20 mM KCl, as indicated at the top of the figure. After addition of $K^+$ in the phosphorylation mixture, PKC phosphorylation of the 19-kDa fragment and the M5/M6 hairpin (12 kDa) is increased. (*A, lower frame*) Immunoblot of the same samples probed by the C-terminal specific Na,K-ATPase antibody indicating a constant amount of 19-kDa fragments in the preparations. (*B*) The stoichiometry of phosphorylation of the 19- and 12-kDa fragments as a function of the $K^+$ concentration. PKC phosphorylation of the 19-kDa fragment and the M5/M6 hairpin (12-kDa) is stimulated ~2-fold in the presence of 15–20 mM $K^+$. Results represent pmoles of $P_i$ incorporated into 1 $\mu$g of protein. Representative of two independent experiments is shown.

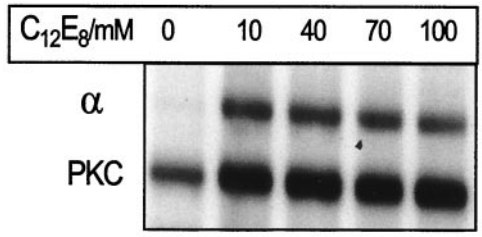



FIGURE 9 PKC phosphorylation of pig kidney Na,K-ATPase in the presence of increasing concentrations of $C_{12}E_8$. Autoradiogram showing PKC phosphorylation of membrane-bound pig kidney Na,K-ATPase (0 mM $C_{12}E_8$), and of enzyme solubilized with increasing $C_{12}E_8$ concentrations as indicated at the top of the figure. The membranes were preincubated with $C_{12}E_8$ and 2 $\mu$l aliquots of the solubilized enzyme were added to the PKC phosphorylation mixture (50 $\mu$l), as described in Materials and Methods. The mixtures were separated by SDS-PAGE (12% Laemmli gels). After autoradiography of the dry gels, the bands corresponding to the $\alpha$-subunits were excised from the gel and the radioactivity measured in a scintillation counter. The phosphorylation stoichiometry was calculated as previously described (Cornelius and Logvinenko, 1996). The calculated phosphorylation stoichiometry (mean ± SE) was 0.020 ± 0.005 (0 mM $C_{12}E_8$), 0.197 ± 0.002 (10 mM $C_{12}E_8$), 0.190 ± 0.003 (40 mM $C_{12}E_8$), 0.156 ± 0.050 (70 mM $C_{12}E_8$), and 0.156 ± 0.020 (100 mM $C_{12}E_8$). Representative of at least three independent measurements is shown.

the phosphorylation mixture as shown by the autoradiogram (Fig. 8 *A*, upper panel). This increase is not due to an increase of the 19 kDa fragment at the different conditions, as indicated by the immunoblot shown in Fig. 8 *A*, lower panel, and could not be produced by addition of 100 mM $Na^+$ to the phosphorylation medium (not shown). The increase in PKC phosphorylation is ~200% and saturates at ~15–20 mM $K^+$ (Fig. 8 *B*). This indicates that conformational changes at the level of the transmembrane segments are important for the phosphorylation at these sites.

## Comparison with pig kidney Na,K-ATPase

The $\alpha$-subunit of pig kidney Na,K-ATPase lacks the main N-terminal PKC phosphorylation site, Ser-18 (Feschenko and Sweadner, 1995), which explains that PKC-phosphorylation to a nondetectable level in membrane-bound pig enzyme (labeled 0 mM $C_{12}E_8$) was found, as demonstrated in Fig. 9. However, as in shark enzyme, detergent treatment increased the phosphorylation level of the pig enzyme, but in contrast to the shark enzyme, the maximum level was only ~0.15 mol $P_i$/mol $\alpha$ and did not increase further by increasing detergent concentration (Fig. 9). It is possible that the conformational changes produced by increasing $C_{12}E_8$ concentrations are opposed by its denaturing effect in pig enzyme preventing an increase in PKC-phosphorylation. In contrast to shark Na,K-ATPase, the pig kidney enzyme is highly sensitive to $C_{12}E_8$, being completely inhibited at ~200 $\mu$M detergent.

We have previously demonstrated that the 19-kDa fragment is phosphorylated in the presence of detergent (Mahmmoud and Cornelius, 2000). In the present investigation these results were further complemented to demonstrate whether the M5/M6 hairpin of the pig $\alpha$-subunit was phosphorylated by PKC in the presence of detergent such as the shark enzyme. Fig. 10 *A* shows the results of PKC phosphorylation of intact or M5/M6-devoid 19-kDa membranes prepared from pig kidney enzyme by incubating in the presence or absence of $K^+$ ions followed by washing. As indicated, a 12-kDa fragment representing the M5/M6 fragment was also phosphorylated by PKC in a detergent-dependent manner (Fig. 10 *A*).

## Detergent-activated PKC phosphorylation of the $\gamma$-subunit

As also seen from Fig. 10 *A*, a strong phosphorylation by PKC of a fragment of apparent molecular mass of 8 kDa was noticed in addition to the 19-kDa and 12-kDa fragments. Phosphorylation of this fragment also required TX-100 (Fig. 10 *A*). It is clear that phosphorylation of this site could not be located at the M5/M6 fragment because it migrates significantly faster in the gel than the M5/M6 fragment, and it is phosphorylated with the same efficiency whether or not the M5/M6 was present in the membranes. Phosphorylation of the shark $\alpha$-subunit at this molecular mass range has not been observed (Fig. 7), suggesting that

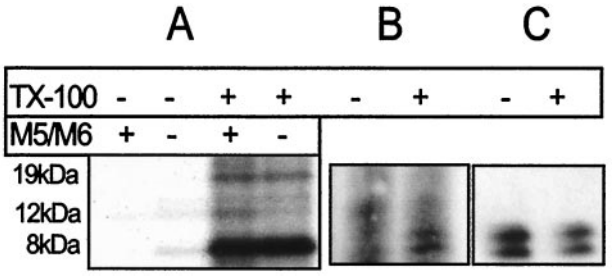



FIGURE 10 Detergent-mediated PKC phosphorylation of a site located in the M5/M6 hairpin of the pig kidney $\alpha$-subunit and in the $\gamma$-subunit. *A*. An autoradiogram showing PKC phosphorylation of the pig kidney 19-kDa membranes incubated in a Tris-buffer (to obtain release of the M5/M6 fragment, labeled M5/M6−) or in a $K^+$-buffer (labeled M5/M6+), as indicated at the top of the figure. The 19-kDa fragment was phosphorylated whether or not the M5/M6 hairpin was present in the membranes. The 12-kDa band is phosphorylated only in the presence of detergent. Another band running at ~8–10 kDa, which was not noticed in shark enzyme (Fig. 8 *A*) is shown in panels *B* and *C* to represent the $\gamma$-subunit. Representative of two independent experiments is shown. For details see Materials and Methods. (*B*) An autoradiogram of a PVDF membrane showing PKC phosphorylation of the 8-kDa $\gamma$-subunit in the absence (*left*) or in the presence of TX-100 (*right*) under controlled conditions of low concentration of [$^{32}$P]ATP (0.75 $\mu$Ci/pmol) and extended exposure time to show the $\gamma$-doublet. The SDS-gel was stained with a solution containing 10% ethanol for only 20 min to avoid extraction of the $\gamma$-subunit from the gel. (*C*) Immunoblot showing the same samples as in (*B*) probed with a $\gamma$-specific antibody.

this site is present only in the pig enzyme. To characterize this site further, the phosphorylation-state sensitive antibodies were used, and it was found that the 8-kDa fragment is phosphorylated at a serine, and not at a threonine residue (not shown). Moreover, SDS-PAGE of Na,K-ATPase, where PKC phosphorylation was performed by incubation with a low concentration of radioactive ATP in the presence of detergents followed by autoradiography, demonstrated the presence of a phosphorylated doublet (Fig. 10 *B*), typical of the $\gamma$-subunit. Finally, as shown in Fig. 10 *C*, the bands were probed after immunoblotting using an anti-$\gamma$ antibody (kindly provided by Dr. S.J.D. Karlish).

To calculate how much phosphate is incorporated into the $\gamma$-doublet after PKC phosphorylation, 2 $\mu$g of pig kidney membrane protein (containing 4.4 pmol $\alpha$, as measured from phosphorylation experiments) was phosphorylated in the presence of detergent. Analysis of the autoradiograms showed that 1.06 $\pm$ 0.1 pmol $P_i$ is incorporated into the $\gamma$-doublet. Assuming that the $\alpha$- and the $\gamma$-subunits are expressed in stoichiometric amounts, a stoichiometry of ~0.24 mole $P_i$/mole $\gamma$-subunit can be estimated. In comparison, PKC phosphorylation of pig $\alpha$ rarely exceeds 0.15 mole $P_i$/mole $\alpha$ (Fig. 9).

## DISCUSSION

In the present study several independent observations demonstrate the presence of C-terminal PKC phosphorylation sites in addition to the conventional N-terminal sites in at least two different Na,K-ATPase $\alpha$-isoforms. First, the N-terminal truncated $\alpha$-subunit, where the conventional N-terminal PKC sites are removed by mild proteolysis, can be phosphorylated to near stoichiometric levels by PKC in the presence of detergents (Fig. 2). Second, proteolytic fingerprinting of PKC-phosphorylated Na,K-ATPase in the presence of detergent demonstrated that several of the C-terminal proteolytic fragments, as detected by C-terminal specific $\alpha$-antibody, were phosphorylated by PKC (Fig. 3 *B*, *lower panel*). Third, by using phosphorylation-sensitive antibodies, it could be demonstrated that the N-terminal truncated $\alpha$-subunit of shark enzyme is phosphorylated by PKC at a threonine residue in the presence of TX-100 (Fig. 6 *A*, *top panel*). Finally, a 12-kDa PKC substrate was identified as the M5/M6 hairpin of the $\alpha$-subunit, as detected by autoradiography (Fig. 7) and immunoblots using a phosphoserine-specific antibody (Fig. 6 *A*, *lower panel*). Common to these newly identified C-terminal PKC-sites are their predicted close proximity to the membrane and the need for detergents to access them, at least in vitro.

Actually, phosphoamino acid analysis has previously shown that PKC phosphorylated both serine and threonine residues in the $\alpha$-subunit from shark rectal glands (Bertorello et al., 1991) and from duck salt glands (Chibalin et al., 1992). Differential phosphorylation at multiple sites may also account for the high stoichiometries found in some studies (Bertorello et al., 1991), as it is possible that different experimental manipulations could lead to the exposure of otherwise inaccessible PKC sites. This also regards experiments where PKC phosphorylation is measured after previous PKA phosphorylation in which detergents were used (Cheng et al., 1997), a condition that could expose C-terminal PKC sites. Finally, that PKC sites other than the conventional N-terminal ones can be accessed in vivo is also suggested by experiments where deletion of the N-terminal PKC sites did not completely abolish phorbol-12-myristate-13-acetate (PMA)-stimulated phosphorylation of Na,K-ATPase in intact cells (Beguin et al., 1994).

### Multisite kinase phosphorylation of a membrane-transport protein

The complexity of protein kinase regulation of Na,K-ATPase has recently been further emphasized by the suggestion that apparent interaction between the PKA and PKC signaling pathways in the phosphorylation of Na,K-ATPase may exist (Feschenko et al., 2000). Such interactions have been inferred from findings of modulation of the PKC phosphorylation of Na,K-ATPase by phosphorylation at the PKA site (Borghini et al., 1994; Cheng et al., 1997). In general, cross-talk between the PKA and PKC pathways is to be anticipated because many membrane transport proteins contain sites for both PKA and PKC (West et al., 1991; Li et al., 1992; Jensen et al., 1993; Krarup et al., 1998).

Interplay between the PKA and PKC phosphorylation of the cystic fibrosis transmembrane conductance regulator has been reported to modulate its function (Jia et al., 1997). Unless allosteric in nature the structural basis for such cross-talk has been difficult to reconcile regarding the Na,K-ATPase because the conventional phosphorylation sites for PKA (Ser-938, rat $\alpha$1) and PKC (Ser-11 and Ser-18) seem spatially widely separated, as deduced from the presumably homologous 3-D structure of the Ca-ATPase (Toyoshima et al., 2000). However, the presence of a conserved PKC phosphorylation sites only four amino acids upstream the PKA site in the same M8/M9 loop may form the functional basis for such cross-talk between the PKA and PKC signaling pathways. PKC-phosphorylation at the M8/M9 loop exposing the Ser-942 to PKA could be one example of achieving such cross-talk between the two signaling pathways. This emphasizes the importance of precise identification of the 3-D topology of protein kinase phosphorylation sites on the Na,K-ATPase $\alpha$-subunit, as well as their interplay, especially regarding possible multisite regulation (Cohen, 2000).

### Accessibility of membrane-adjacent phosphorylation sites may be subject to physiological control

A fundamental question to address is why detergents are needed to access these C-terminal phosphorylation sites in vitro. The physiological significance of phosphorylation at the PKA site has been controversial (Feschenko et al., 2000), because in vitro phosphorylation of membrane-bound Na,K-ATPase by PKA requires the presence of detergents such as TX-100 (Chibalin et al., 1992, 1993; Feschenko and Sweadner, 1994). The same reservations could apply to the newly identified C-terminal PKC sites reported in this investigation.

The requirement for detergents to access these sites could suggest that some structural organizations constrain their accessibility in vitro. Recent studies attempting to model the spatial location of the PKA motif from the homologous 3-D structure of the Ca-ATPase do seem to indicate a location of the PKA phosphorylation site close enough to the plasma membrane to make it inaccessible to the kinase (Sweadner and Feschenko, 2001). However, it is unknown to what degree the crystal structure of the Ca-ATPase, presumably in the E1-conformation, reflects the in vivo structure of the Na,K-ATPase. Moreover, Na,K-ATPase can be phosphorylated at the Ser-938 site in the absence of detergents both in vivo (Carranza et al., 1996; Cheng et al., 1997; Kiroytcheva et al., 1999) as well as in vitro after reconstitution (Cornelius and Logvinenko, 1996).

That the inaccessibility of the PKA site to the kinase should be attributable to structural constraints including crowding by the nearby C-terminal tail and the partial overhanging of the N or P domains of the $\alpha$-subunit (Sweadner and Feschenko, 2001) does not seem to be supported by the present investigation. Extensive proteolysis of membrane preparations removing any major cytoplasmic structural constraints was found inadequate to expose the PKA site (Fig. 4 *B*). Furthermore, PKC phosphorylation of the closely shaved 19-kDa fragment at the same M8/M9 loop still required the presence of detergent (Fig. 4 *A*). This indicates that is not the burial of, or shielding by, cytoplasmic domains per se that prevents the phosphorylation of these membrane-adjacent sites. Rather, exposure of these sites may be a membrane-dependent process or a process dependent on enzyme conformation. The latter is supported by the observation that $K^+$ ions specifically promote phosphorylation of these fragments by PKC (Fig. 8), indicating that a conformational change toward the E2-like conformation promotes the phosphorylation of these sites. The effect of detergents is probably also attributable, in part, to a shift toward the E2 conformation (Fig. 3).

Why are the C-terminal PKC sites found at positions close to the membrane in loops that exhibit unusually high flexibility? After stimulation, PKC is translocated to the membrane where diacylglycerol, phosphatidylserine, and/or $Ca^{2+}$ activate it, depending on isoform. This serves to bring the enzyme into close proximity with its specific protein substrate. Thus, the regulation of Na,K-ATPase by protein kinase phosphorylation most likely involves internal protein kinase receptors to target and anchor the protein kinases to their substrate sites on the Na,K-ATPase $\alpha$-subunit (Mochly-Rosen, 1995; Faux and Scott, 1996; Pawson and Scott, 1997; Mochly-Rosen and Gordon, 1998; Edwards and Scott, 2000). These processes as well as their components are almost entirely unknown for the Na,K-ATPase.

The M8/M9 loop is known to be extremely structurally flexible as demonstrated by heat denaturation experiments (Karlish et al., 1991; Arystarkhova et al., 1995) in which this loop is even externalized from the membrane upon heating at 55°C. Also, the M5/M6 hairpin has been demonstrated to be very flexible (Lutsenko et al., 1995; Gatto et al., 1999). Such flexibility could be important for controlling the exposition of the phosphorylation sites located within these motifs to accommodate contact to PKC, i.e., the exposure of these sites could in itself represent a regulatory mechanism. In support of this, thermoinactivation of Na,K-ATPase has been demonstrated to increase the PKC phosphorylation level in duck salt gland (Chibalin et al., 1993) and in shark enzyme by increasing the temperature from 24°C to 37°C during the phosphorylation reaction (this study, not shown). Thus, if targeting via receptor proteins that will anchor PKC to the specific protein substrate in the membrane is defective in the purified preparation, detergents are essential to expose the sites to PKC. Kurihara et al. (2000) have recently reported the participation of an A-kinase anchoring protein of molecular mass 150 kDa (AKAP-150) in the phosphorylation and inhibition of Na,K-ATPase

in basolateral membrane vesicles from rat parotid gland acinar cells.

Functional PKC phosphorylation at sites close to the cytoplasmic face of the plasma membrane has previously been found in vivo for the epidermal growth factor receptor (Hunter et al., 1984) and for the receptor-like protein-tyrosine phosphatase (Tracy et al., 1995). Moreover, very similar topological location of PKC sites as found in the present investigation has been found for the $Na^+/HCO_3^-$ co-transporter, including both N-terminal sites as well as C-terminal sites very close to the membrane face (Romero et al., 1997).

### PKC phosphorylation of the $\gamma$-subunit

The $\gamma$-subunit is an ancillary small protein expressed mainly in kidney that has regulatory functions for the Na,K-ATPase $\alpha$1-subunit (Therien and Blostein, 2000). It contains a conserved PKC phosphorylation motif with a serine residue close to the predicted cytoplasmic membrane face. Similar PKC consensus motifs are present in several members of the FXYD-family, including phospholemman.

The present investigation demonstrates that the $\gamma$-subunit can be phosphorylated by PKC to a stoichiometry of $\sim$0.24 mol $P_i$/mol $\gamma$ in the presence of TX-100, the same low stoichiometry as found for pig renal Na,K-ATPase $\alpha$-subunit. Any functional consequences of this phosphorylation on the Na,K-ATPase has yet to be investigated. However, we have previously demonstrated that PKC phosphorylation of shark rectal Na,K-ATPase membranes results in a partial dissociation of another FXYD protein, the phospholemman-like protein from shark that is specifically associated with the Na,K-ATPase, leading to activation (Mahmmoud et al., 2000). Although the topological arrangement of the PKC phosphorylation site of the $\gamma$ is different from that of phospholemman-like protein from shark, the present demonstration that the $\gamma$-subunit can be phosphorylated by PKC could indicate that the interaction of these small single membrane spanning regulatory proteins of the FXYD-family with the Na,K-ATPase may be controlled by kinase phosphorylation. Thus, protein kinase phosphorylation seems not to be a conformational switching signal, but rather a signal to induce coupling/decoupling between proteins, as with tyrosine kinases (Barinaga, 1999).

To summarize, we have demonstrated that at least two novel PKC sites exist in the C-terminal part of shark rectal and pig renal Na,K-ATPase $\alpha$-subunit in addition to the conventional N-terminal sites. These novel sites are highly conserved and characterized by their location close to the plasma membrane. Similar to the PKA site, detergents are needed to induce phosphorylation in vitro; that is suggested because of functional impairment of targeting devices in the purified preparation. The enhanced exposure of the C-terminal PKC sites in the presence of $K^+$ indicate that the exposure of these sites are under physiological control, a fact that is further supported by the high degree of flexibility of the M5/M6 and M8/M9 segments where the sites are located. The existence of several PKC sites may in part explain the complex physiological regulation of this membrane protein by multisite phosphorylation (Cohen, 2000). Finally, the identification of a PKC site in the same M8/M9 loop where the PKA site is located may form the structural basis for cross-talk between the PKA and PKC signaling pathways.

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