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Membrane Enzyme Systems Responsible for the Ca^{2+}-Dependent Phosphorylation of Ser^{27}, the Independent Phosphorylation of Tyr^{10} and Tyr^{7}, and the Dephosphorylation of These Phosphorylated Residues in the α-Chain of H/K-ATPase[1]

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H/K-ATPase preparations (the G1 membrane) from pig stomach contain both kinases and phosphatases and show reversible phosphorylation of Tyr^{7}, Tyr^{10}, and Ser^{27} residues of the α-chain of H/K-ATPase. The Tyr-kinase is sensitive to genistein and quercetin and recognized by anti-c-Src antibody. The Ser-kinase is dependent on Ca^{2+} ($K_{0.5}$ = 0.9 μM), sensitive to a PKC inhibitor, and recognized by antibodies against PKCα and PKCβII. The addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonic acid (CHAPS) caused a dramatic increase in the phosphorylation of added synthetic copolymer substrates and permitted the phosphorylation of maltose-binding proteins fused with the N-terminal domain of α-chains. The phosphotyrosine phosphatase was inhibited by vanadate. The phosphoserine phosphatase was inhibited by okadaic acid and by inhibitor-2. The presence of protein phosphatase-1 was immunologically detected. Column chromatographic separation of CHAPS-solubilized G1 membrane and others indicate the apparent molecular weight of the Src-kinase to be ~60 kDa, the PKCα and/or PKCβII to be ~80 kDa, the Tyr-phosphatase to be 200 kDa, and PP-1 to be ~35 kDa. These data show that these membrane-bound enzyme systems are in sufficiently close proximity to be responsible for reversible phosphorylation of Tyr^{7}, Tyr^{10}, and Ser^{27} of the catalytic subunit of membrane H/K-ATPase in parietal cells, the physiological role of which is unknown.

Key words: H/K-ATPase, kinase, phosphatase, reversible N-terminal phosphorylation.

H/K-ATPase, which is found in parietal cell, plays a major role in gastric acid secretion. It has been reported that several receptor-mediated pathways regulate gastric acid secretion. Histamine stimulates acid secretion *via* H2 receptor–mediated activation of cAMP-dependent protein kinases (PKA) (*1–3*). Acetylcholine also stimulates acid secretion by increasing protein kinase C (PKC) activity *via* an increase in intracellular calcium concentration (*4, 5*). Recent studies suggest that acid secretion requires the presence of cAMP and calcium at a certain level (*6, 7*). The PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibits histamine- and carbachol-induced acid secretion (*8–10*). In the resting state and in dibutyryl cAMP-stimulated gastric glands, TPA enhances acid secretion (*10*). KN-62, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, specifically inhibits carbachol-stimulated acid secretion (*11*).

Tyr-kinase modifiers, epidermal growth factor (EGF), and transforming growth factor–α (TGF-α) inhibit histamine- and carbachol-induced acid secretion (*12–14*) but chronically enhance acid secretion (*13*). Furthermore, pretreatment of parietal cells with genistein, a Tyr-kinase inhibitor, leads to the recovery of TGF-α inhibition (*14*). These data suggest that protein phosphorylation and dephosphorylation are in some way connected with acid secretion.

It has been unequivocally shown that pig stomach H/K-ATPase preparations (G1 membrane fraction) contain kinases and phosphatases which are responsible for the reversible phosphorylation of Tyr^{10}, Tyr^{7} (*15*), and Ser^{27} (*16*) of the H/K-ATPase α-chain. However, the role of Ca^{2+} in Tyr and Ser phosphorylation and the enzymes involved have not been studied in detail, except that the Ser^{27} was also phosphorylated by extrinsic rat brain PKC with Ca^{2+} and PKA (*16*). We recently demonstrated that the N-terminal Tyr residues were phosphorylated *in vivo*, when gastric tissues were incubated with pervanadate (*17*). Characterization of enzyme systems responsible for the reversible phosphorylation of H/K-ATPase are important to understand the molecular mechanism of acid secretion. The present

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Abbreviations: Ser(P), phosphoserine; Tyr(P), phosphotyrosine; PTP, protein tyrosine phosphatase; PVDF, polyvinylidene difluoride, GST, glutathione *S*-transferase, TCA, trichloroacetic acid, TPCK, tosylphenylalanyl chloromethyl ketone, MES, [2-(*N*-morpholino)ethanesulfonic acid], BES, [*N,N*-bis(2-hydroxyethyl)-2 aminoethane sulfonic acid]; CBB, Coomassie Brilliant Blue, MBP, maltose-binding protein, HRP, horseradish peroxidase.

data show that a c-Src, Ca^{2+}-dependent PKCs, transmembrane type Tyr-phosphatase, and protein phosphatase 1 are present in the membrane and in sufficiently close proximity to be responsible for reversible phosphorylation of Tyr^{7}, Tyr^{10}, and Ser^{27} of the N-terminal domain of catalytic subunit of membrane H/K-ATPase.

MATERIALS AND METHODS

Vesicles containing pig stomach H/K-ATPase (G1 membrane fraction) were prepared and stored in the presence of 250 mM sucrose and 0.5 mM EGTA-Tris, pH 7.4 at –80°C (*18*). The specific activity of the H/K-ATPase preparations was around 120 μmol/h/mg at 37°C. The protein concentration of the enzymes was determined by the method of Bradford with bovine serum albumin (Pierce) as a standard (*19*). P_i was determined as described previously (*20*). Phosphoamino acid analysis of the phosphorylated α-chain was performed as described previously (*15*).

To follow calcium-dependent phosphorylation, 0.4 mg protein/ml of G1 membrane was incubated at 30°C for 5 min with buffer containing 50 mM BES-NaOH (pH 7.1), 10 mM $MgCl_2$, 7.4% sucrose, 2 mM [γ-^{32}P]ATP, and various concentrations of $CaCl_2$ using Ca-EGTA buffer in a final volume of 50 μl. In order to follow Tyr phosphorylation, BES-NaOH and $CaCl_2$ were replaced with 50 mM HEPES-Tris (pH 7.4), 1 mM vanadate, and 2 mM dithiothreitol. The reaction was stopped by the addition of SDS-sample buffer. Samples were analyzed by electrophoresis using 10% polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*21*). After separation, the gels were stained with Coomassie brilliant blue (CBB), then destained. The incorporation of ^{32}P into proteins was analyzed by a Bioimage analyzer BAS 2000 (Fuji). For immunodetection, radioactive ATP was omitted. After SDS-PAGE, proteins were blotted to Immobilon (0.45 μm, Millipore). The blots were first incubated with specific antibodies, developed by SuperSignal™ Substrate (Pierce), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The relative intensity of each band was quantitated by an NIH image after scanning the X-ray film. The phosphorylation of fusion proteins bound to maltose-binding protein and synthetic polymer poly(Arg/Ser) or poly(Glu/Tyr) were done as described (*15*) except that CHAPS-solubilized membranes (see below) were used. For the determination of the inhibition by genistein, the ATP concentration was reduced to 50 μM at a protein concentration of 0.05 mg/ml for 5 min.

For solubilization, 3% CHAPS was added to a suspension of 4.8 mg protein G1 membrane/ml of buffer containing 40 mM HEPES-Tris (pH 7.4), 2 mM EGTA-Tris, 2 mM EDTA-Tris, leupeptin, aprotinin and pepstatin A (5 μg/ml each), and 2 mM β-mercaptoethanol with stirring at 0°C for 30 min. For gel filtration and immunoprecipitation, the sample was centrifuged at 100,000 rpm for 10 min (Optima TL, 100.3 rotor, Beckman), and the supernatant was collected as a CHAPS supernatant.

For *in vitro* kinase assay, the CHAPS supernatant was first incubated with specific antibodies in a binding buffer (50 mM HEPES-Tris, pH 7.4, 5 mM EDTA-Tris, and 3% CHAPS), then the complex was adsorbed to protein A-agarose. After washing the gel with binding buffer, MBP-HK was phosphorylated as described above in the absence of dithiothreitol.

Total mRNA was extracted from pig stomach using the TRIsol reagent (BRL) and converted to cDNA by reverse transcriptase (SuperScript, BRL) with oligo(dT) as a template primer. cDNA corresponding to the N-terminal 110 amino acids from Gly^{2} to Gln^{111}, the numbers of which correspond to the initiation methionine as residue 1 of the α-chain of H/K-ATPase, was amplified by the PCR method using specific synthetic oligonucleotide primers (5′-GGG AAG GCG GAG AAT TA-3′ and 5′-CAC ATA TGT CAC TGC AGA CCG CCC GC-3′ for the forward and reverse primers, respectively). The amplified cDNA fragments were ligated to pMAL vector. The sequence analysis was performed by the dideoxy method (Sequenase, USB). The N-terminal domain was fused to the maltose-binding protein, then expressed in *Escherichia coli* by isopropyl thiogalactoside induction. Recombinant protein was purified by amylose resin by the standard procedure. For the construction of mutated fusion proteins, the megaprimer PCR mutation method was used to introduce mutations changing Tyr^{7} and Tyr^{10} to Phe^{7} and Phe^{10}. GST-fusion proteins were constructed as described previously (*17*).

The CHAPS supernatant was applied to a column of dual-connected Superose 12 (1 cm × 25 cm, Amersham Pharmacia Biotech) equilibrated with an elution buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM β-mercaptoethanol, and 250 mM sucrose containing 0.7% CHAPS]. Fractions of 0.5 ml were collected at a flow rate of 20 ml/h at 0°C and analyzed for Tyr-kinase activity using the fusion protein as a substrate. Reactivities against anti-c-Src kinase antibodies, SRC2 (polyclonal, Santa Cruz), and GD11 (monoclonal, Upstate Biotechnology), were detected by Western blotting.

[γ-^{32}P] ATP was purchased from Amersham Pharmacia Biotech. Type-specific anti-PKC antibodies and protein A-agarose were purchased from Santa Cruz. PKC inhibitor was obtained from RBI. Genistein was obtained from extrasynthese. Quercetin was purchased from Wako Chemicals. Poly(Arg/Ser) and poly(Glu/Tyr) were obtained from Sigma, and human recombinant c-Src was purchased from Upstate Biotechnology. All other chemicals were of the highest grade commercially available.

RESULTS

Effect of Ca^{2+} on the N-Terminal Phosphorylation—It has been shown that the incubation of 1.5 mM Ca^{2+} with the G1 membrane in the presence of ATP for 90 min increased phosphorylation of both Ser and Tyr residues of N-terminal α-chain of H/K-ATPase (*16*). However, the time course of the phosphorylation showed that the amount of Ser(P) increased initially, then decreased with time (not shown). To investigate the role of Ca^{2+} in the phosphorylation, the initial phase of α-chain phosphorylation in the presence of various concentrations of Ca^{2+} was measured after 5 min. The addition of Ca^{2+} without or with TPA increased the incorporation to ~1.6-fold ($K_{0.5}$ = 0.9 μM) and 2.1-fold ($K_{0.5}$ = 0.6 μM) (Fig. 1A). The value for 100% phosphorylation is taken as the value in the presence of EGTA without addition of Ca^{2+}: free Ca^{2+} concentration was estimated to be ~3 nM by fluorescence measurements using Fura-2. A PKC inhibitor abolished Ca^{2+}-dependent phosphorylation. Mild

TPCK-trypsin treatment completely removed both Ca^{2+}-dependent and independent radioactivity present in the ~100 kDa peptide. The data indicate that phosphorylation by intrinsic kinases occurred at Ser^{27}, Tyr^{10}, and Tyr^{7} of the α-chain of H/K-ATPase, as has previously been shown (*15, 16*).

The addition of CHAPS somewhat reduced the α-chain phosphorylation and permitted the phosphorylation of Tyr and Ser residues of low molecular weight proteins (20–60 kDa) in the G1 membrane (*22*). The effect of CHAPS on the phosphorylation of Ser residues was investigated with changing the concentrations of Ca^{2+}. CHAPS abolished the Ca^{2+}-dependent increase almost completely, while TPA opposed it (Fig. 1B). CHAPS increased the extent of inhibition by the PKC inhibitor. Phosphoamino acid analysis indicated that Ca^{2+} increased the content of Ser(P) ~3-fold independently of the presence of CHAPS and/or TPA (Table I). These data are consistent with the role of Ca^{2+} in the Ser phosphorylation by a conventional PKC. The effect of TPA on the Ca^{2+}-dependent phosphorylation of the α-chain was rather small in the absence of CHAPS. These findings can be explained by assuming that CHAPS removed phosphatidylserine and diacylglycerol, which are required for the intrinsic PKC activity in the presence of Ca^{2+}.

Phosphorylation of Synthetic Polymer Substrates by the G1 Membrane—To investigate whether G1 membrane kinases are capable of catalyzing the phosphorylation of synthetic substrates, the membrane was incubated with a Ser-kinase substrate poly(Arg/Ser) in the presence of 100 μM Ca^{2+} or with a Tyr-kinase substrate poly(Glu/Tyr) in the presence of vanadate. The presence of the poly(Arg/Ser) and poly(Glu/Tyr), respectively, increased the incorporation of ^{32}P into the acid-insoluble fraction of G1 membranes and copolymers to 2- and 7-fold (Table II). CHAPS caused a slight reduction in the incorporation into the Ser and Tyr residue of the α-chain in the absence of copolymers and, thus, had a negligible influence on the reactions. However, the simultaneous presence of CHAPS and each copolymer increased the net phosphorylation of each synthetic polymers by a factor of 3–4-fold (Table II).

Phosphorylation of Fusion Proteins Containing N-Terminal Domain of H/K-ATPase—Preliminary experiments showed that kinases in the G1 membrane were not able to phosphorylate the fusion proteins made from the maltose-binding protein and a part of N-terminal α-chain containing Gly^{2} to Gln^{111} (MBP-HK) with or without Ca^{2+}, unless CHAPS was present. It has already been shown that CHAPS permitted the phosphorylation of the low molecular weight proteins in the G1 membrane and synthetic copolymers (*17, 22*). To investigate this point further, CHAPS-treated G1 was further incubated with increasing concentrations of the fusion protein. Figure 2 shows only a slight decrease in the extent of α-chain phosphorylation with increases of Tyr and Ser phosphorylation of MBP-HK, respectively, in the presence of vanadate and Ca^{2+}. These data suggest that kinases present in the G1 membrane had considerably higher affinities for α-chains than for MBP-HK, even in the presence of CHAPS: a 10-fold excess of MBP-HK on a molar basis inhibited α-chain phosphorylation only ~20% in the presence of vanadate and Ca^{2+}, respectively.

Effect of an Activator and an Inhibitor on the Phosphorylation of Fusion Proteins—TPA and a PKC inhibitor in-

TABLE I. **Phosphoamino acid analyses of the α-chain.** Phosphorylation was carried out as described under "MATERIALS AND METHODS" Phosphoamino acid analysis was performed as described previously (*15*).

CHAPS-treatment	Condition	Total (%)	Tyr(P)	Ser(P)
–	EGTA	100	59.1	40 9
–	Ca^{2+}	204	70.2	134
+	EGTA	85 0	54 2	30 8
+	Ca^{2+} + TPA	198	96 4	102

TABLE II **Incorporation of ^{32}P into synthetic polymers.** Poly (Arg/Ser), 0 25 mg/ml, was phosphorylated with 0.1 mg/ml of G1 or CHAPS-treated G1 membrane fraction in a buffer containing 25 mM BES-NaOH (pH 7 1), 100 μM $CaCl_2$, 10 mM $MgCl_2$, 2 mM DTT, 648 nM TPA, and 500 μM ATP for 5 min In the case of poly (Glu/Tyr) phosphorylation, BES-NaOH, $CaCl_2$, and TPA were replaced with 50 mM HEPES-Tris (pH 7.4) and 1 mM Na_3VO_4.

	poly(Arg/Ser)		poly(Glu/Tyr)	
	G1	G1 + CHAPS	G1	G1 + CHAPS
	(pmol)		(pmol)	
–poly	40 1 ± 0.3	25 6 ± 2 2	11 7 ± 1 4	11 8 ± 1.6
+poly	80.1 ± 5.1	284 ± 3 3	84.6 ± 2 1	232 ± 0 9

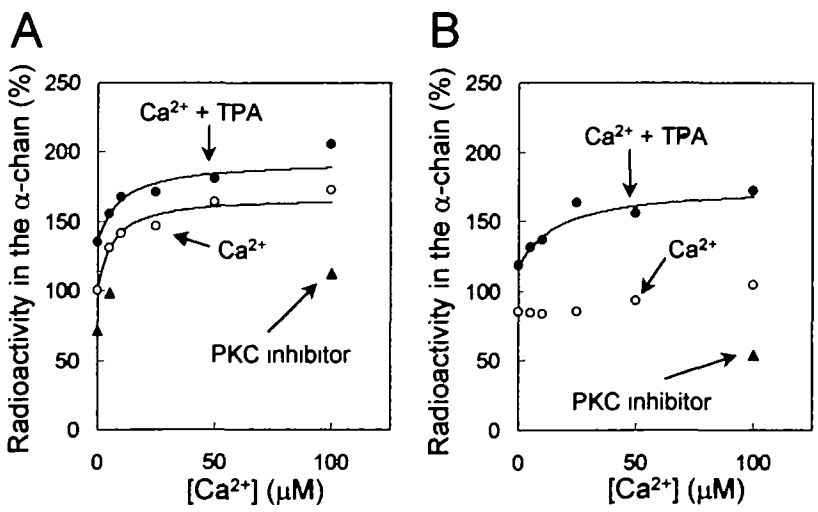

Fig. 1 **Effect of Ca^{2+} concentration on phosphorylation of the α-chain of the H/K-ATPase in G1 membrane.** G1 membranes (A) or CHAPS-treated G1 membranes (B) were phosphorylated with various concentrations of Ca^{2+} without (○) or with 648 nM TPA (●) or with 100 μM PKC inhibitor (▲) for 5 min The reaction was stopped by the addition of SDS-sample buffer, and samples were subjected to SDS-PAGE. After staining with CBB, incorporation of radioactivity into the α-chain was analyzed by BAS 2000 One hundred percent value represents the radioactivity of the α-chain in the G1 membrane without added Ca^{2+}. The data shown in this paper are the typical examples of the means of triplicate experiments.

creased and decreased α-chain phosphorylation, respectively, in the G1 membrane in the presence of CHAPS (Fig. 1B). To confirm that the effects are the result of the phosphorylation of Ser[27], a mutant fusion protein ($Y^{7,10}F$) was used, in which both Tyr[10] and Tyr[7] were replaced with Phe to delete Tyr-kinase-dependent phosphorylatable sites.

The G1 membrane was incubated with the $Y^{7,10}F$ in the presence of 100 μM Ca^{2+}. Phosphorylation was detected in the α-chain (~100 kDa) and the $Y^{7,10}F$ (~57 kDa) as shown in Fig. 3. The presence of CHAPS was also prerequisite for the phosphorylation of the fusion protein, which was accompanied by a reduction in α-chain phosphorylation. The PKC inhibitor nearly completely inhibited phosphorylation of the $Y^{7,10}F$ in the presence of CHAPS. A significant reduction in α-chain phosphorylation by the inhibitor would be due to the reduction of the Ser phosphorylation, and the remaining phosphorylation would be due to the phosphorylation of Tyr by an intrinsic Tyr-kinase. The effect of TPA appeared to be a slight increase in the phosphorylation of both substrates in the presence of CHAPS (see also Fig. 1B). The addition of either cAMP, cGMP, or calmodulin had no effect on the phosphorylation of the α-chain nor that of the $Y^{7,10}F$ by intrinsic Ser kinase in the G1 fraction (not shown). These data strongly suggest that the intrinsic membrane-bound conventional PKC phosphorylates Ser[27] of the α-chain.

Two different Tyr-kinase inhibitors, genistein and quercetin, strongly inhibited Tyr(P) formation in the α-chain, as detected by an anti-Tyr(P) monoclonal antibody. The inhibition by genistein was reversed by high concentrations of ATP (Fig. 4A). Divalent cations, such as 10–20 mM Mg^{2+} or 3–4 mM Mn^{2+} induced the maximum phosphorylation of poly(Glu/Tyr), as has already been shown with a purified c-Src (*23*).

Recognition of Protein Kinases by Antibodies—To characterize protein kinases present in the G1 membrane, their reactivity against antibodies to protein kinases was examined. Calcium ion-dependent phosphorylation of Ser[27] by intrinsic Ser kinase suggested the presence of conventional PKC in the G1 membrane. When purified PKC from rat brain was added to the G1 membrane, Ser[27] was also phosphorylated in the presence of Ca^{2+} + TPA. Western blotting analysis showed that two bands at 80 and 47.5 kDa reacted with antibodies against PKCα and -βII but not -βI or -γ (Fig. 5A). The $Y^{7,10}F$ was also phosphorylated by these antigen-antibody-protein A-agarose complexes ob-tained from a CHAPS supernatant of the G1 membrane (Fig. 5B). The data showed that a conventional PKC, which was present in the G1 membrane, was responsible for Ca^{2+}-dependent phosphorylation of Ser[27] in the H/K-ATPase α-chain.

The Tyr-kinase seemed to be associated with the membrane and was inhibited by specific inhibitors (Fig. 4) and activated by Mg^{2+} or Mn^{2+}. These data and the apparent molecular weight of the kinase as described later (Fig. 7A) suggested the presence of a Src-kinase in the membrane. The Western blotting analysis showed that several protein bands, 60–50 kDa on SDS gel, reacted with polyclonal (SRC2) and monoclonal (GD11) anti-c-Src antibody (Fig. 6A), while no bands reacted with anti-c-Fyn and anti-c-Yes (not shown). When the G1 membrane was preincubated

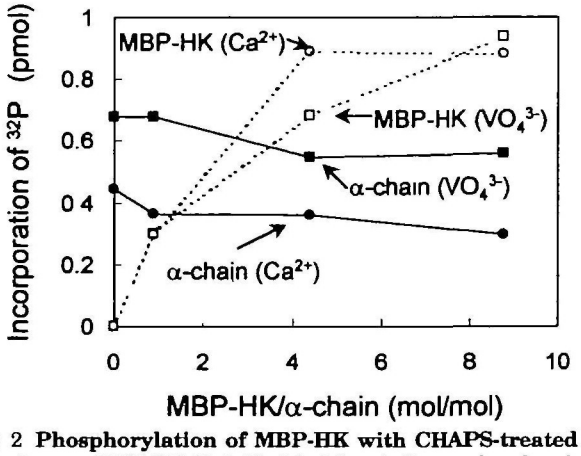

Fig 2 **Phosphorylation of MBP-HK with CHAPS-treated G1 membrane.** MBP-HK (0, 0.02, 0.1, 0.2 μg/ml) was phosphorylated with 0.04 μg/ml CHAPS-treated G1 membrane with 1 mM Na_3VO_4 (squares) for 90 min or with 0.25 mM $CaCl_2$ (circles) for 20 min. The reaction was stopped by the addition of SDS-sample buffer, and aliquots of samples were subjected to SDS-PAGE. After staining with CBB, incorporation of radioactivity into the α-chain (closed) and MBP-HK (opened) was analyzed by BAS 2000

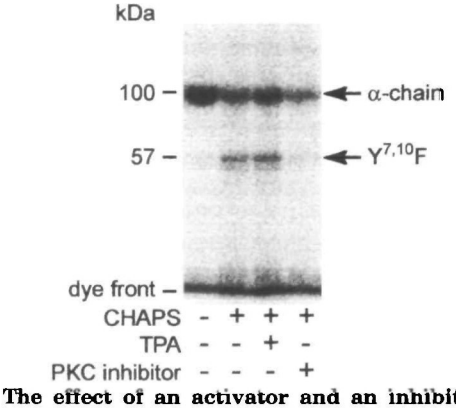

Fig. 3 **The effect of an activator and an inhibitor on the phosphorylation of $Y^{7,10}F$.** $Y^{7,10}F$ (0.1 mg/ml) was phosphorylated with G1 membrane, which had been pretreated with or without CHAPS as described as Fig 1 The samples were separated by SDS-PAGE and analyzed by BAS 2000

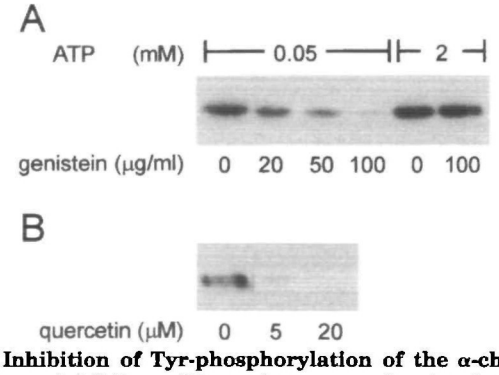

Fig. 4 **Inhibition of Tyr-phosphorylation of the α-chain by Tyr-kinase inhibitors.** G1 membrane was phosphorylated with various concentrations of genistein (A) with 0.05 or 2 mM ATP, or quercetin (B) with 0 1 mM ATP for 5 min. The samples were separated by SDS-PAGE and the proteins were transferred to PVDF membranes. Blots were analyzed for reactivity of anti-Tyr(P) antibody and developed by SuperSignal™ Substrate followed by incubation with HRP-conjugated secondary antibody

with an anti-c-Src antibody, SRC2, amounts of Tyr(P) in the α-chain decreased with increasing amounts of antibody as shown in Fig. 6B. The antigen and antibody complex obtained from solubilized G1 showed phosphorylating activity for Tyr residues of MBP-HK, which was detected with anti-Tyr(P) monoclonal antibody (Fig. 6C). MBP-HK was also phosphorylated by the human recombinant c-Src (not shown).

The amount of Src-kinase in the G1 membrane was estimated from the amount of antibody bound to the enzyme using a recombinant c-Src as a standard. The data indicated that the amount of Src kinase present was ~1 μg Src protein/mg protein of the G1 protein, or <~1 mol/400 mol of α-chain (Fig. 6D). This suggests that only a catalytic amount of Src kinase is present in the membrane, and that the enzyme must diffuse rather rapidly in order to phosphorylate another α-chain of H/K-ATPase in the sequence.

Column Chromatographic Separation of Solubilized Kinases—It has been shown that CHAPS solubilized both Tyr- and Ser-kinases, which are present in the G1 membrane. The solubilized fraction was subjected to gel filtration to estimate the molecular mass of these kinases. Aliquots of each fraction were used to measure activities of Tyr-kinase and Ser-kinase in the presence of poly(Glu/Tyr) and poly(Arg/Ser), respectively, as shown in Fig. 7A. Two main peaks having Tyr-kinase activity were detected in fractions 28 and 30, while the Ser-kinase activity was resolved in two clearly separated peaks in fractions 22 and 28. Western blotting analyses with an anti-c-Src antibody showed the maximum amount of the 60 kDa protein was present in fraction 27 or 28, while that of 50 kDa protein was present in fraction 30 (Fig. 7B). The former fractions also induced maximum phosphorylation of MBP-HK, which was detected by an anti-Tyr(P) antibody, but the latter fraction gave little Tyr(P) (Fig. 7C). The data suggest that a 60 kDa Src-kinase is responsible for the phosphorylation of Tyr^{10} and Tyr^{7} of H/K-ATPase α-chain.

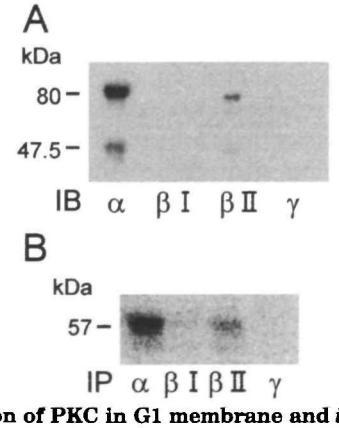

Fig. 5. **Detection of PKC in G1 membrane and *in vitro* kinase assay for phosphorylation of fusion proteins.** (A) G1 membrane (10 μg) was separated by SDS-PAGE and the blot was analyzed by Western blotting with anti-conventional PKC isoforms antibodies. (B) The supernatant of CHAPS-treated G1 membrane was incubated with each isoform antibody, and the immune complexes were recovered by protein A-agarose. After washing, the gel suspension was incubated with $Y^{7,10}F$ in the buffer containing 50 mM HEPES-Tris (pH 7.4), 10 mM $MgCl_2$, 15 mM NaF, 0.6 mM $CaCl_2$, 648 nM TPA, and 2 mM [γ-^{32}P]ATP at 30°C for 60 min. The samples were separated by SDS-PAGE and analyzed by BAS 2000.

Western blotting analysis of the PKCs of each fraction suggested that the maximum amount of PKCα and PKCβII, respectively, was present in around fractions 28 or 29 and 27–29 (Fig. 7, D and E). These data suggest that 80 and 60 kDa proteins reacted with anti-PKC and c-Src antibody, respectively, and are responsible for the phosphorylation of N-terminal Ser^{27}, Tyr^{10}, and Tyr^{7} residues in H/K-ATPase α-chain.

Characterization of Phosphatases in the G1 Membrane—The reversible phosphorylation of both Ser and Tyr residues of N-terminal H/K-ATPase suggest the presence of phosphatases in the G1 membrane. The addition of vanadate increased the phosphorylation, which suggests the accumulation of Tyr(P) due to the inhibition of Tyr phosphatase in the membrane (*15*, *17*). The time course of the Ca^{2+}-dependent Ser(P) accumulation showed an initial increase followed by a decrease, as described above. These data suggest the presence of a Ser-phosphatase. The effect of Ser-phosphatase inhibitors on the dephosphorylation of GST-HK[Ser(P)] by the G1 membrane showed that the presence of 1 and 100 nM of okadaic acid led to a reduction in the activity to 63 and 20% level, respectively. The addition of inhibitor-2 also reduced the activity to ~20%. The

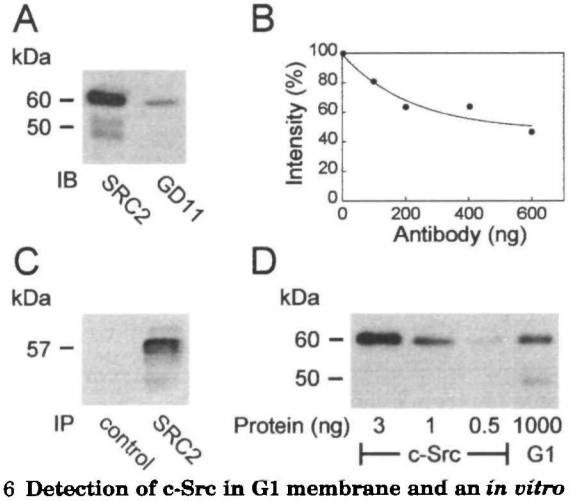

Fig. 6 **Detection of c-Src in G1 membrane and an *in vitro* kinase assay for phosphorylation of fusion proteins.** (A) G1 membrane (4 μg) was separated by SDS-PAGE and the blot was analyzed by Western blotting with anti-c-Src polyclonal antibody, SRC2, or monoclonal antibody, GD11 (B) G1 membrane (0.4 mg/ml) was preincubated with SRC2 in the phosphorylation mixture without ATP for 10 min at 0°C for 60 min. Phosphorylation was initiated by the addition of ATP. After incubation for 5 min, samples were separated by SDS-PAGE and proteins were blotted to PVDF membrane. Tyr phosphorylation was detected by Western blotting with anti-Tyr(P) antibody The intensity of anti-Tyr(P) antibody-reactive α-chain was estimated by NIH image. The intensity of Tyr phosphorylated α-chain of G1 membrane preincubated with each amount of non-immune IgG was set as 100%. (C) The supernatant of CHAPS-treated G1 membrane was incubated with SRC2, and the immune complex was recovered by protein A-agarose. After washing, the gel suspension was incubated with MBP-HK (WT) in the buffer containing 50 mM HEPES-Tris (pH 7.4), 10 mM $MgCl_2$, 15 mM NaF, 1 mM Na_3VO_4, and 2 mM ATP at 30°C for 60 min Tyr phosphorylation was detected by Western blotting with an anti-Tyr(P) antibody. Non-immune IgG was used as a control. (D) The amount of c-Src in the G1 membrane fraction (1 μg) was estimated by Western blotting using SRC2. Purified human recombinant c-Src was used as a standard.

presence of Ca^{2+}, Ca^{2+} + calmodulin, or Mg^{2+} showed little effect on the Ser-phosphatase activity. Western blotting analysis showed (*24*) the presence of PP1δ, but not PP1α, PP2A, or PP2B.

It has been shown that the dephosphorylation of Tyr^{10}(P), Tyr^{7}(P), and Ser^{27}(P) of the H/K-ATPase α-chain which were phosphorylated by intrinsic kinases in the G1 membrane or extrinsic kinases is accelerated by added G1 membrane which had not been preincubated with ATP. These data suggest that phosphatases present in the membrane are able to break down phosphorylated α-chains which are present in another membrane. Repeated washing of the G1 membrane by centrifugation had a negligible effect on the phosphatase activities of the G1 membrane. Although enzymes of the PP1 family are assumed to be soluble, the data suggest that phosphatases involved, including PP1δ, were associated with the G1 membrane. To solubilize these phosphatases, CHAPS was added to the G1 membrane. CHAPS strongly inhibited the breakdown of Ser(P) of GST-HK but had no significant effect on that of Tyr(P) of GST-HK. The breakdown of the Tyr(P) of GST-HK by the G1 membrane was accelerated up to two orders of magnitude by 1% Triton X-100 (Fig. 8), suggesting that the solubilization increased the accesibility of the Tyr-phosphatase to a soluble substrate, GST-HK[Tyr(P)].

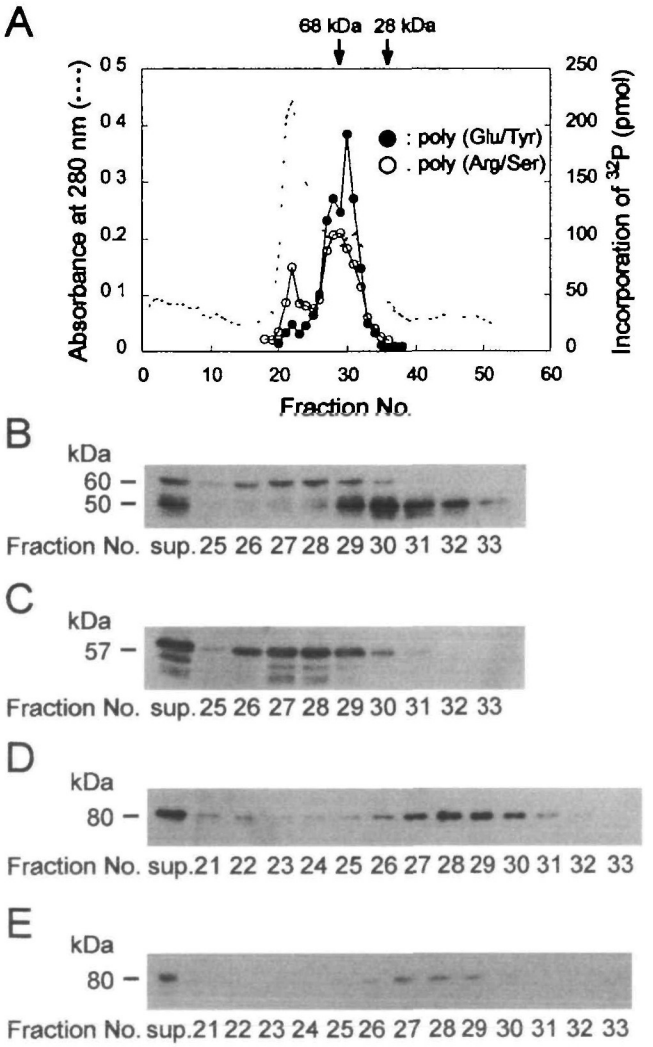

Fig. 7. **Partial separation of solubilized kinases.** (A) G1 membrane (4.8 mg/ml) was solubilized with 3% CHAPS and ultracentrifuged. The supernatant was applied on dual-connected columns of Superose 12 (1 × 25 cm) equilibrated with the elution buffer. The activity to phosphorylate poly(Glu/Tyr) (●) and poly(Arg/Ser) (○) and optical density (---) were measured in each fraction. Standard proteins, BSA (68 kDa) and GST (28 kDa), were used as indicated. Reactivity to SRC2 (B), anti-PKCα (D), and anti-PKCβII (E) antibodies in each fraction (indicated in the figure) was analyzed by Western blotting. Supernatant was used as a control (C). The activity to phosphorylate MBP-HK (WT) was measured in each fraction. The samples were separated by SDS-PAGE and blots were analyzed by Western blotting with anti-Tyr(P) antibody.

It has been reported that membrane-bound Tyr-phosphatases show vanadate sensitive *p*-nitrophenyl phosphate (pNPP) hydrolysis (*25*) in the presence of EDTA. To investigate whether this also occurs in the Tyr-phosphatase of the G1 membrane, the hydrolysis of pNPP was measured in the presence of EDTA with or without vanadate and/or CHAPS. The presence of CHAPS increased the activity ~200%, while the presence of vanadate decreased the activity ~10% independently of the presence of CHAPS.

Gel Filtration Chromatography of Solubilized Phosphatases—When the H/K-ATPase preparation was solubilized by 3% CHAPS and the soluble fraction was subjected to Sephacryl S-300 gel filtration chromatography, three peaks which showed pNPPase activity were detected. The majority of the total pNPPase activity (>85%) was recovered in the first peak fractions (Fig. 9A). GST-HK[Tyr(P)] was dephosphorylated only by these peak fractions, and not by the second and third peak fractions (Fig. 9B). The apparent molecular mass of the first peak was in excess of 200 kDa, and the increase in the activity for the extrinsic substrate by solubilization suggests that this Tyr phosphatase is of the transmembrane type (*26*). The cross-reactivity of CHAPS-solubilized H/K-ATPase preparations (G1 membrane) to several antibodies against transmembrane type Tyr phosphatases [leukocyte common antigen-related (LAR), PTPβ, PTPδ, PTPγ, PTPκ, PTPμ] was investigated. The presence of LAR and PTPδ was detected in the G1 membrane (not shown). However, the elution profiles of the CHAPS-solubilized sample of the dephosphorylating activity of the GST-HK[Tyr(P)] on Sephacryl S-300 column chromatography were different from those of LAR and PTPδ (not shown).

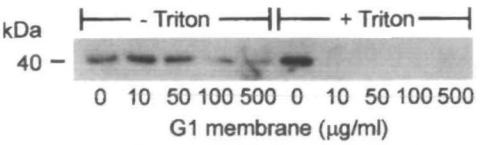

Fig 8 **Dephosphorylation of GST-HK[Tyr(P)] by membrane-bound Tyr phosphatase.** Tyr phosphorylated GST-HK was prepared as shown in Fig 6C and desalted by dialyzing against 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 2 mM β-mercaptoethanol. The G1 membrane (2 mg/ml) was pretreated with or without 1% Triton X-100 in buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA-Tris, 2 mM EGTA-Tris, and 2 mM β-mercaptoethanol at 0°C for 30 min. GST-HK[Tyr(P)] (60 μg/ml) was incubated with G1 membrane pretreated with or without Triton X-100 in buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM DTT, and 5 mM EDTA-Tris at 30°C for 5 min. Dephosphorylation of GST-HK[Tyr(P)] was analyzed by Western blotting with anti-Tyr(P) antibody.

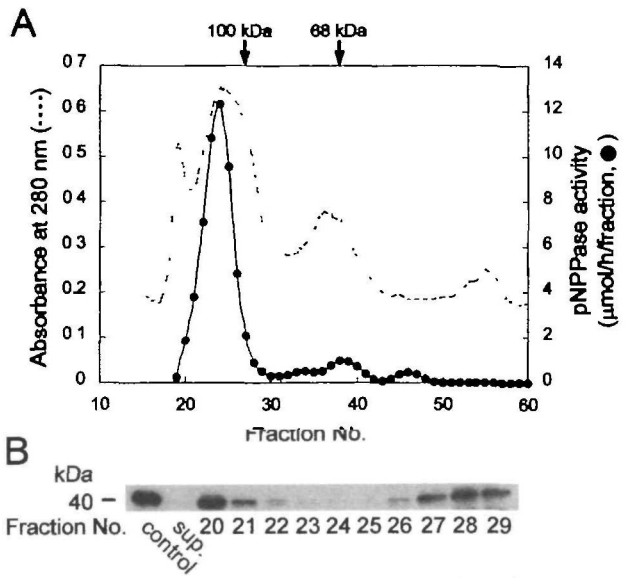

Fig. 9. **Estimation of molecular mass of Tyr phosphatase which dephosphorylate N-terminal Tyr(P) of the H/K-ATPase α-chain.** (A) G1 membrane (4.8 mg/ml) was treated with 3% CHAPS and ultracentrifuged The resulting supernatant was applied to Sephacryl S-300 (1 6 × 60 cm, Amersham Pharmacia Biotech) equilibrated with the elution buffer, and the samples were eluted at a flow rate of 30 ml/h. The activity of pNPPase (●) and optical density (---) were measured in each 2-ml fraction. The pNPPase activity was assayed in a buffer containing 50 mM MES-Tris (pH 5 5), 10 mM DTT, 5 mM EDTA-Tris, 10 mM pNPP-Tris at 30°C for 20 min. The reaction was stopped by the addition of 4 volumes of 1 N NaOH, and pNP was quantitated by absorption at 410 nm. Standard proteins, H/K-ATPase α-chain (100 kDa) and BSA (68 kDa), were used as indicated (B) The activity to dephosphorylate the N-terminal domain of the H/K-ATPase in each fraction was measured by dephosphorylation of GST-HK[Tyr(P)] Tyr phosphorylated GST-HK (control) was incubated with 20-fold-diluted supernatant or each fraction in a buffer shown in Fig. 8 for 10 min Dephosphorylation of GST-HK[Tyr(P)] was analyzed by Western blotting with an anti-Tyr(P) antibody

DISCUSSION

Ca^{2+} Dependent and Independent Phosphorylation—Ser phosphorylation of the α-chain was increased by addition of Ca^{2+} (Fig. 1 and Table I). Western blotting analysis, studies using an inhibitor and an activator, and an *in vitro* kinase assay showed that PKC was present in the G1 membrane and induced the Ca^{2+}-dependent phosphorylation of Ser^{27} of the α-chain (Fig. 5) Tyr-phosphorylation occurred in the presence of EGTA (free Ca^{2+} concentration <2 nM) (Fig. 1 and Table I). Western blotting analyses, inhibitor studies, and an *in vitro* kinase assay showed that a c-Src was present in the G1 membrane and induced the Ca^{2+} independent phosphorylation of Tyr^{10} and Tyr^{7} of the α-chain.

The 60 kDa SRC2-reactive protein phosphorylated the N-terminal domain of H/K-ATPase, namely, Tyr^{10} and Tyr^{7} (Fig. 7). While the 50 kDa fraction did not, the relative amount of 60 and 50 kDa proteins varied from preparation to preparation and the content of 50 kDa protein increased after solubilization. The N-terminal portion of the c-Src peptide from residue 10 to 80 is the most variable among the Src family kinases, contributing to their biological specificity (*27*). A c-Src was proteolitically cleaved at the N-terminal side to give 52–54 kDa protein (*28, 29*). These data are consistent with the hypothesis that the ~50 kDa SRC2-reactive protein represents a cleavage product of the 60 kDa kinase, produced by a protease which is present in the G1 membrane (*16, 17, 30*).

The present data strongly suggest that a c-Src and PKC are each present in sufficiently close proximity to phosphorylate Tyr^{10} and Tyr^{7} and Ser^{27} of H/K-ATPase α-chain, respectively, in the absence and presence of Ca^{2+}: externally added substrates were phosphorylated only after the addition of CHAPS. The Ca^{2+}-dependent and independent phosphorylation may indicate some presently unknown physiological role in the H^+ and K^+-transport system. It was not possible to isolate α-chain in which both Tyr and Ser residues are phosphorylated in H/K-ATPase, and which might be related to the oligomeric nature of the enzyme (*20, 31*).

Physiological Role of Reversible Phosphorylation—There appear to be at least two different explanations for the role of the reversible phosphorylation of H/K-ATPase. One is the change in the H/K-ATPase activity, which is detectable in the broken membrane preparation; and the other is not directly related to the activity but, rather, to H^+ and K^+-transport, which is detectable in tight vesicles or parietal cells. We were not able to detect any significant changes in the enzymatic activity of the G1 membrane after phosphorylation (*15*). However the phosphorylation of Ser residue in other P-type ATPases has been reported to change the activity. The phosphorylation of Ser^{23} of the Na/K-ATPase α-chain by PKC (*32*) and phosphorylation of Ser^{38} of cardiac Ca-ATPase by Ca^{2+}/calmodulin-dependent protein kinase, respectively, have been reported to reduce the apparent affinity for K^+ and to increase the V_{max} for Ca^{2+}-transport 2-fold (*33*).

Recently some relationships between reversible phosphorylation and vesicle traffic on P-type ATPase have been reported. Dopamine-induced endocytosis of the Na/K-ATPase, internalization of Na/K-ATPase into intracellular components, is reported to be initiated by phosphorylation of Ser^{18} in the α-chain (*34*). It has also been reported that the H/K-ATPase-containing cytoplasmic tubulovesicles are recruited into the apical plasma membrane upon stimulation and recycled into the cytoplasm in resting states (*35*). In fact, Rab11 and Rab25, small GTP-binding proteins that are thought to regulate a number of aspects of vesicle trafficking, are present in the H/K-ATPase-containing vesicles (*36*). Further studies will be required to clarify the relationship between the reversible phosphorylation of the N-terminal domain of the H/K-ATPase and the vesicle trafficking.

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