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Phosphorylation of liver gap junction protein by protein kinase C

Akira Takeda, Eikichi Hashimoto+, Hirohei Yamamura+ and Takashi Shimazu

Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu, Ehime 791-02 and +*Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11, Japan*

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The 27 kDa protein, a major component of rat liver gap junctions, was shown to be phosphorylated in vitro by protein kinase C. The stoichiometry of the phosphorylation indicated that approx. 0.33 mol phosphate was incorporated per mol 27 kDa protein. Phosphorylation was entirely dependent on the presence of calcium and was virtually specific for serine residues. For comparison, the gap junction protein was also examined for its phosphorylation by cAMP-dependent protein kinase, the extent of phosphorylation being one-tenth that exerted by protein kinase C.

Gap junction; Protein kinase C; cyclic AMP; Protein phosphorylation; (Rat liver)

1. INTRODUCTION

Gap junctions are channels that permit directly cell-to-cell communication via the passage of ions and small molecules between contacting cells [1]. The gap junction membrane can be isolated from liver by subcellular fractionation following alkali treatment and its constituent protein has been shown to be 27 kDa as determined by SDS-PAGE [2]. Each channel is thought to be a dodecamer of this 27 kDa protein, a hexamer forming the hemichannel in each of the joined membranes [3].

Recently, inhibitory effects of phorbol ester and diacylglycerol on cell-to-cell communication have been reported in cultured cells [4,5]. It is known that phorbol ester and diacylglycerol are direct activators of protein kinase C, and that protein kinase C plays a crucial role in signal transduction for a variety of biologically active substances that activate many cellular functions and proliferation [6]. It thus seems possible that protein kinase C directly inhibits the permeability of the gap junction by phosphorylating its constituent 27 kDa protein.

In support of this possibility, we report here that the 27 kDa gap junction protein is actually phosphorylated in vitro by protein kinase C.

Correspondence address: A. Takeda, Dept of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu, Ehime 791-02, Japan

Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Gap junction membranes were purified from rat liver as described by Hertzberg [2] with a modification of the treatment with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) before alkali extraction. The purity of gap junction preparations was assessed by both negative stain electron microscopy and SDS-PAGE. The amino acid composition of the preparations was also analyzed as in [7].

Protein kinase C was purified and assayed according to Kitano et al. [8] (spec. act. 500 nmol/min per mg protein). Cyclic AMP-

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dependent protein kinase was purified from bovine heart as described by Rubin et al. [9] and assayed according to Takai et al. [10] (spec. act. 168 nmol/min per mg protein).

Phosphorylation of gap junction membranes was carried out at 30°C for 30 min. Where indicated in the figure legends, 1.5 mM $CaCl_2$ and/or phospholipid (4 μg phosphatidylserine and 0.4 μg diolein) were added to the reaction mixture of 0.5 ml (final volume) containing 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 10 μM [γ-^{32}P]ATP (2–3 Ci/mmol), 0.025% Triton X-100, 0.25 mM EDTA, 0.25 mM EGTA, 5 mM 2-mercaptoethanol, 5% glycerol and purified gap junction membranes (2.8 μg protein) in the presence or absence of protein kinase C (5 μg). Under similar conditions, phosphorylation of gap junction membranes (2.8 μg protein) by cAMP-dependent protein kinase (100 μg) was also carried out at 30°C for 30 min in a final volume of 0.5 ml containing 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, 10 mM magnesium acetate and 10 μM [γ-^{32}P]ATP with or without 1 μM cAMP. The reaction was stopped by the addition of 0.125 ml of 0.2 M EDTA (pH 7.5). Then, gap junction membranes were collected by centrifugation at 10000 × g for 10 min, resuspended in 0.8 ml of water and recentrifuged. The pellets were incubated with Laemmli sample buffer [11] at room temperature for 30 min. SDS-PAGE was carried out on 12.5% polyacrylamide gel by the method of Laemmli [11] with Coomassie blue staining. Molecular mass standards used were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). After drying the gel, autoradiography was carried out by using X-ray film (Fuji JX). Bands of the gap junction protein were excised from the dried gel, solubilized overnight in 30% H_2O_2 at 50°C, and their radioactivities determined by liquid scintillation counting [12].

Phosphoamino acid was analyzed according to Kumon et al. [13]. Gel pieces corresponding to the 27 kDa protein were extracted and hydrolyzed in 6 N HCl for 2 h at 110°C. The hydrolyzed materials mixed with phosphoamino acid standards were subjected to high-voltage paper electrophoresis at 50 V/cm for 1.5 h with formic acid-acetic acid-water (24:87:889), pH 1.9.

Protein concentration was determined by the method of Bradford [14] with bovine serum albumin as a standard. [γ-^{32}P]ATP (7000 Ci/mmol) was purchased from ICN Radiochemicals. All other chemicals were of analytical grade.

3. RESULTS AND DISCUSSION

The purity of gap junction membranes used in this experiment was quite satisfactory for both morphological and biochemical criteria [15]. The intrinsic protein of purified gap junction membranes gave two major bands of 27 kDa and 50 kDa on SDS-PAGE (fig.1A, lane 1). It has been shown that a major component of the liver gap junction is the 27 kDa protein and that the 50 kDa band is an aggregated dimer of the 27 kDa protein in the presence of SDS [15]. Moreover, the amino acid composition of the gap junction preparations corresponded to the results reported in [7,16,17] (not shown).

When purified gap junction membranes were incubated with protein kinase C, phosphate incorporation took place specifically into the gap junction protein (27 kDa protein and its 50 kDa dimer) as revealed by SDS-PAGE and autoradiography (fig.1A,B, lane 2). This phosphorylation was entirely dependent on the presence of calcium. Omission of Ca^{2+} from the reaction mixture resulted in a drastic decrease in phosphorylation of the gap junction protein (fig.1B, lane 3). However, no additional increase was observed in the degree of phosphorylation of the gap junction protein by further addition of phospholipid to the reaction mixture (fig.1B, lane 4), and addition of phospholipid also increased both membrane-bound protein kinase C and its corresponding autophosphorylation (fig.1A,B, lane 4). Probably because purified gap junction membranes contain phospholipids sufficient for binding and activation of protein kinase C, it seems difficult to demonstrate phospholipid-dependent phosphorylation of the gap junction protein. Omission of protein kinase C resulted in virtually no phosphorylation of the gap junction protein, indicating that purified gap junction membranes did not contain contaminating kinase activities (fig.1B, lane 1).

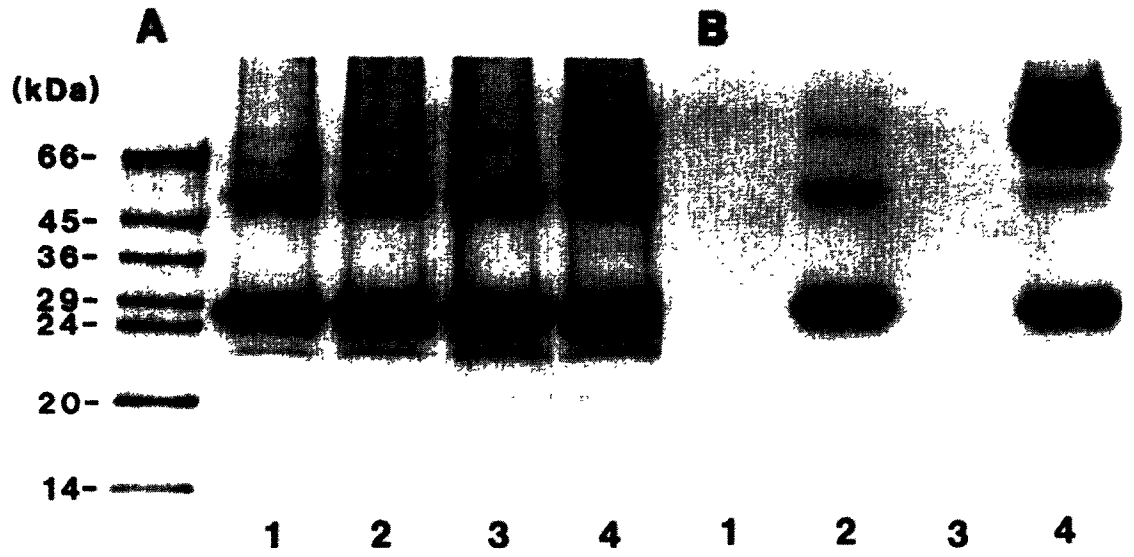

Fig.1. Phosphorylation of the 27 kDa protein in purified gap junction membranes by protein kinase C. A Coomassie blue-stained gel (A) and its corresponding autoradiogram (B) are shown. Gap junction membranes (2.8 μg protein) were incubated for 30 min with [γ-^{32}P]ATP in either the absence (lane 1) or presence (lanes 2–4) of protein kinase C (5 μg). $CaCl_2$ and/or phospholipid were added to the reaction mixture as follows: lanes: 1,2, $CaCl_2$ (1.5 mM); 3, none; 4, $CaCl_2$ plus phospholipid (4 μg phosphatidylserine and 0.4 μg diolein).

Fig.2 shows the time course of phosphorylation of the 27 kDa protein by protein kinase C. In the presence of calcium, the stoichiometry of phosphorylation indicated that approx. 0.33 mol phosphate was incorporated per mol 27 kDa protein in 60 min. For comparison, phosphorylation of gap junction membranes by cAMP-dependent protein kinase was also examined under conditions similar to those described in section 2. The radioactivities of gel pieces corresponding to the 27 kDa protein were 30156 cpm for protein kinase C and 2982 cpm for cAMP-dependent protein kinase, respectively, determined by Cerenkov radiation. Although the phosphate incorporation by cAMP-dependent protein kinase was relatively low, this reaction proceeded in a cAMP-dependent manner. These results showed that the degree of phosphorylation of the 27 kDa protein by protein kinase C was approx. 10-fold greater than that by cAMP-dependent protein kinase.

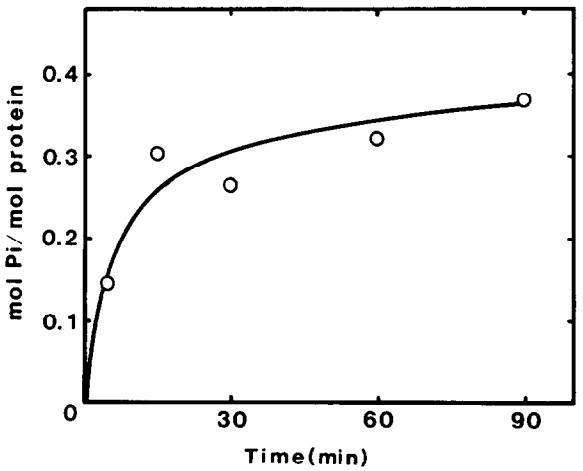

Fig.2. Time course of phosphorylation of the 27 kDa protein. Phosphorylation was determined in terms of radioactivity of the gap junction protein extracted from the gel after SDS-PAGE as described in section 2.

Fig.3 shows the phosphoamino acid analysis of the phosphorylated 27 kDa gap junction protein. Phosphorylation of the 27 kDa protein by cAMP-dependent protein kinase occurred only in serine residue. When the 27 kDa protein was phosphorylated by protein kinase C, only phosphoserine was also detected, whereas little phosphothreonine could be found.

A number of specific treatments have been found to affect the properties of the gap junction,

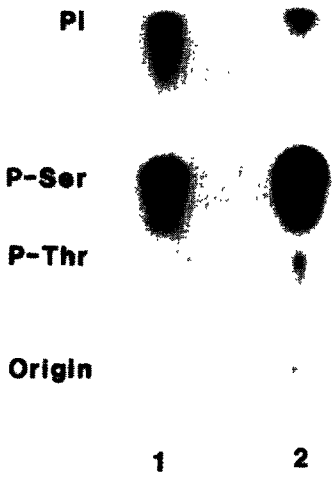

Fig.3. Phosphoamino acid analysis of the 27 kDa protein phosphorylated and isolated by SDS-PAGE followed by autoradiography. The autoradiogram is shown. The 27 kDa protein was phosphorylated by cAMP-dependent protein kinase (lane 1) and by protein kinase C (lane 2) as described in section 2. P-Ser, phosphoserine; P-Thr, phosphothreonine.

some of which are involved in the short-term regulation of its permeability [1]. Recently, it has been shown that cAMP increases junctional permeability as well as phosphorylation of the gap junction protein [18]. On the other hand, either phorbol ester or diacylglycerol is reported to decrease junctional permeability in cultured cells [4,5]. Hence, it has previously been proposed that a dual regulatory system may exist for regulating junctional permeability via the protein kinase C-dependent and cAMP-dependent pathways [5].

From our present results, it becomes apparent that the 27 kDa protein of the gap junction is more effectively phosphorylated by protein kinase C than by cAMP-dependent protein kinase. It was also reported that the 27 kDa protein was phosphorylated with low stoichiometry by the catalytic subunit of cAMP-dependent protein kinase [18], the extent of phosphorylation being comparable to our present data using the holoenzyme.

Although in the present experiments we have no evidence for phosphorylation in vivo, our results suggest that the 27 kDa protein is a relatively specific substrate for protein kinase C. It remains to be elucidated what alteration does occur in the properties of liver gap junctions after phosphorylation by protein kinase C and to be established the physiological significance of this phosphorylation in vivo.

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