Induction of phosphotyrosine in the gap junction protein, connexin43

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Abstract The protein-tyrosine phosphatase inhibitors pervanadate, permolybdate, H_2O_2, and to a much lesser extent vanadate, increased the amount of cellular phosphotyrosine and induced tyrosine phosphorylation of connexin43 (Cx43) in early passage hamster embryo fibroblasts. The presence of phosphotyrosine in Cx43 immunoprecipitates from pervanadate-treated cells was shown by a phosphotyrosine-specific antibody and a phosphotyrosine-specific phosphatase. Pervanadate-induced Cx43 tyrosine phosphorylation was further verified by phosphoamino acid analysis, while no phosphotyrosine was present in control cells. This is the first observation of tyrosine phosphorylation of connexins in normal cells.

Key words: Protein-tyrosine phosphatase inhibitor; Hydrogen peroxide; Pervanadate; Permolybdate; Phosphotyrosine; Connexin

1. Introduction

Gap junctional intercellular communication (GJIC) is assumed to be involved in several fundamental processes like development, differentiation and growth control [1,2], and many signal transduction pathways are known to affect GJIC [3]. Gap junction channel proteins are called connexins. At present, 13 connexin genes have been cloned from mammals. Connexin43 (Cx43) is a major connexin present in many tissues [4]. Most connexins appear to be phosphoproteins or to possess potential phosphorylation sites. Connexins may be targets for several serine/threonine kinases [5–7]. The phosphorylation of Cx43 occurs almost exclusively on serine. There are conflicting results whether there is a rapid turnover of phosphates in Cx43 [8] or not [9]. No phosphatase has been identified as responsible for dephosphorylation of connexins in intact cells, but relatively little work has been devoted to the question. Okadaic acid and similar compounds do not affect GJIC or the phosphorylation status of Cx43 in Syrian hamster embryo (SHE) cells, even after treatment with 12-*O*-tetradecanoylphorbol 13-acetate [10]. However, okadaic acid delays the normalization of Cx43 band pattern in rat liver epithelial cells treated with epidermal growth factor [11]. Vanadate is a classical protein-tyrosine phosphatase (PTPase) inhibitor. Usually, it has little effect on GJIC [10,12,13], but it affects GJIC in cells transfected with a temperature-sensitive variant of v-Src [14]. H_2O_2 is an oxidative agent and a PTPase inhibitor [15,16]. It may slightly inhibit GJIC in some cell types [17,18], but is without effect [19] or may even increase GJIC in other cells [20]. However, pervanadate (a mixture of vanadate and H_2O_2) significantly and potently affects GJIC and the phosphorylation status of Cx43 [10].

Pervanadate is a PTPase inhibitor [15,21] with specificity differing from vanadate [21]. The biological effect of pervanadate was first detected by its insulin mimesis [22], but it also affects a number of other cellular and biochemical functions, such as T-cell activation-like events [23–25], tyrosine phosphorylation and activation of phospholipases Cγ [24,26] and D [27], increased formation of inositol phosphates [26,28], and activation of the transcription factor NF-κB [24].

We have recently characterized the nature of pervanadate [29]. The results indicated that the biologically active compound of pervanadate is diperoxovanadate(V) (see [29] for further references). Also permolybdate is an insulin mimetic [30], it decreases GJIC and alters the Cx43 band pattern in Western blots [31]. Permolybdate is probably diperoxomolybdate [31]. The strong effect of pervanadate and permolybdate on both GJIC and Cx43 phosphorylation in intact cells raised the possibility that these compounds induce tyrosine phosphorylation of Cx43. Up to now, tyrosine phosphorylation of connexins in intact cells has only been detected in cells transfected with oncogenes encoding the activated tyrosine kinases v-Src [8,32,33] and v-Fps [34].

2. Materials and methods

2.1. Chemicals

Na_3VO_4 (sodium orthovanadate, vanadate) was bought from Sigma (St. Louis, MO, USA). H_2O_2 and $Na_2MoO_4 \cdot 2H_2O$ (sodium molybdate) were obtained from Aldrich (Steinheim, Germany).

2.2. Preparation of pervanadate and permolybdate

The concentration of H_2O_2 was controlled by potassium permanganate titration. The metal salts were dissolved in water to 1 M (molybdate) or 300 mM (vanadate). Pervanadate was made by co-incubating 30 mM vanadate and 60 mM H_2O_2. Permolybdate was made by acidifying 100 mM molybdate with 120 mM HCl before addition of 200 mM H_2O_2. The metal salts and H_2O_2 were co-incubated for 15 min (room temperature, in the dark) before addition to the cells. The concentration of the metal ion is regarded as the concentration of the per-compound.

In some experiments the alkaline colorless stock solution of vanadate was neutralized with HCl, giving the yellow-orange 'decavanadate' solution.

2.3. Cell cultures

Primary cell cultures (mainly fibroblasts) from Syrian hamster (Wright, Chelmsford, Essex, UK) embryos (SHE cells) were prepared and cultured as described [10], and used between passages 2 and 10. No differences in responses were found between early and late passages.

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Abbreviations: GJIC, gap junctional intercellular communication; Cx43, connexin43; SHE, Syrian hamster embryo; PTPase, protein-tyrosine phosphatase; Vanadate, orthovanadate; NP, P1, P2, the non-phosphorylated and the major phosphorylated forms of Cx43

A preliminary part of these data was presented at the IV European Congress of Cell Biology, Prague, Czech Republic, 26 June–1 July 1994 [49].

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2.4. Western blotting and detection of phosphotyrosine in Cx43

Western blotting was performed as described [10] applying 10–20 μg protein/lane. Cx43 was detected by an anti-peptide rabbit antiserum [10]. Phosphotyrosine was detected by a phosphotyrosine-specific monoclonal antibody (4G10; Upstate Biotechnology, Inc.).

For immunoprecipitation, the cells were scraped into immunoprecipitation buffer (20 mM Tris pH 9.0, 0.3% (w/v) sodium lauroyl sarcosine, 150 mM NaCl, 1% (w/v) Triton X-100, 20 μg/ml pepstatin A, 20 μM leupeptin, 50 units/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride), sonicated, and pre-cleared with protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Cx43 was immunoprecipitated with anti-Cx43 antiserum and protein A Sepharose CL-4B. The immune complexes were washed 5 times before further treatment. Tyrosine phosphorylation of Cx43 was examined using several methods. (i) The immunoprecipitates were subjected to Western blotting, using 4G10 as the primary antibody. (ii) A recombinant fragment of a phosphotyrosine-specific phosphatase from *Yersinia enterocolitica* (Boehringer Mannheim) was employed to dephosphorylate the immunoprecipitates. The specific activity of the *Yersinia* phosphatase is at least 5 orders of magnitude higher with phosphotyrosine as substrate compared to phosphoserine or phosphothreonine [35]. Dephosphorylation was performed on half of the immunoprecipitate as described by the supplier. The other half was buffer treated. After solubilization in Laemmli sample buffer, each part was again divided into two, one part for blotting with the anti-Cx43 antiserum, the other part for blotting with 4G10. (iii) Immunoprecipitates from ^{32}P-labeled cells (4 h of 1 mCi/60 mm dish followed by 30 min exposure to 30 μM pervanadate or vehicle) were blotted to polyvinylidene difluoride membranes, and the position of labeled Cx43 was identified by autoradiography. The appropriate areas were cut out from the blots, and subjected to acid hydrolysis. Two-dimensional phosphoamino acid analysis was done as described [36].

3. Results

Cx43 from control SHE cells separated into three major bands and some minor bands (Fig. 1, lower panel). The lower major band was the non-phosphorylated (NP) form of Cx43, and the upper bands were phosphorylated forms of Cx43 [10]. As in many other cell types, the arbitrary assignment P1 and P2 will be used for the major lower and upper phosphorylated bands in unexposed cells. A minor species was found just above NP. There was a small amount of phosphotyrosine-containing proteins in control cells (Fig. 1, upper panel), the major species were migrating in positions corresponding to approximately 83, 85, 91, 95, 115, 125 and 160 kDa, and with some minor species between 60 and 72 kDa.

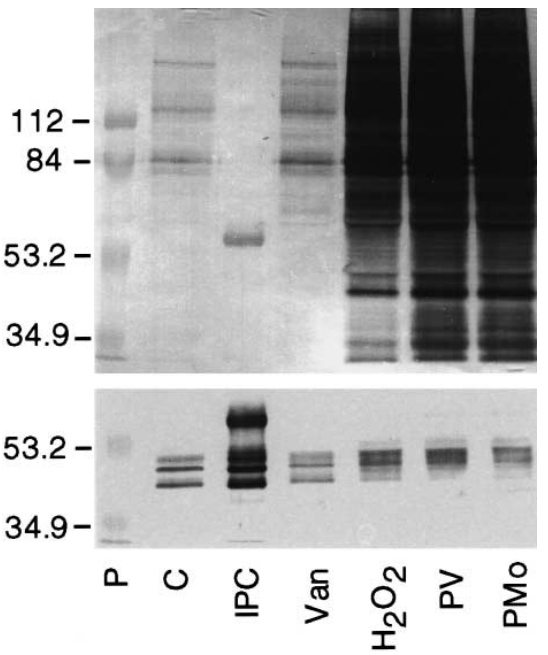

Fig. 1. Induction of cellular tyrosine phosphorylation (upper panel) and alterations in band pattern of Cx43 (lower panel). SHE cells were vehicle-treated (control, C), or exposed to 10 mM vanadate (Van), 3 mM H_2O_2, 300 μM permolybdate (PMo) or 30 μM pervanadate (PV) for 30 min before sampling. Western blots were probed with 4G10 (upper panel) or anti-Cx43 antiserum (lower panel). For comparison, immunoprecipitate from control cells (IPC) is shown. P, prestained standards with calibrated molecular mass in kDa on the left.

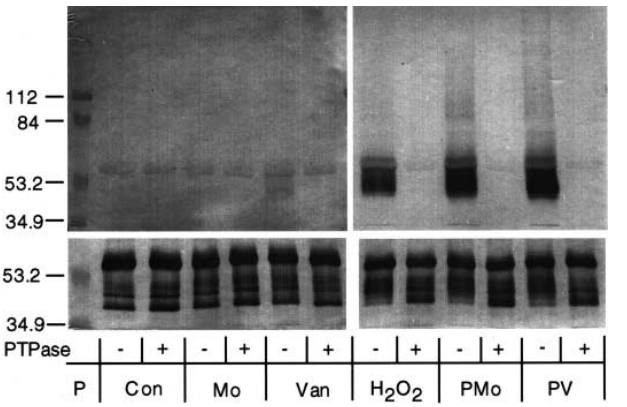

Fig. 2. Detection of phosphotyrosine in Cx43. Cx43 was immunoprecipitated from control cells and cells treated with molybdate (10 mM), vanadate (10 mM), H_2O_2 (3 mM), permolybdate (300 μM) or pervanadate (30 μM) for 30 min. Each of the immunoprecipitates was divided into two, and one of the halves was treated with the phosphotyrosine-specific phosphatase from *Yersinia enterocolitica*. Each half was again divided into two. One part was probed with the anti-Cx43 antiserum (lower panel). The other part was probed with 4G10 (upper panel).

Vanadate gave a small increase in phosphotyrosine and a slight blurring of the Cx band pattern at 3–10 mM (Fig. 1). Vanadate strongly increased the pH of the medium when added at 3 and 10 mM, but a pH shock was not found to mimic the effects of vanadate (not shown). Neutralized vanadate ('decavanadate') seemed to be slightly less potent than vanadate (not shown). H_2O_2 (3 mM) increased cellular phosphotyrosine (Fig. 1, upper panel). The Cx43 band pattern was also changed at 3 mM H_2O_2 (Fig. 1, lower panel). This was characterized by a decrease in the intensity of the NP species of Cx43, and an increased smear in the area of the upper phosphorylated Cx43 bands. It should be noted that the present batch of SHE cells was more sensitive to H_2O_2 than most other cell batches tested (by a factor of 3). Also pervanadate and permolybdate caused increases in cellular phosphotyrosine and prominent changes in the band pattern of Cx43, but at substantially lower concentrations than H_2O_2 (Fig. 1, lower panel). Cx43 immunoprecipitates from control cells showed the same pattern of bands as the corresponding total homogenates (Fig. 1, lower panel), with the exception that P2 often separated into double bands. This difference was probably due to a highly abundant protein migrating immediately above P2 in the total homogenates. The yield of P2 in the immunoprecipitates could vary somewhat (compare with Fig. 2, lower panel). The strong band migrating at approximately 55 kDa was the heavy chain of the immunoglobulins. The heavy chain gave a non-specific reaction in the phosphotyrosine blots (Fig.

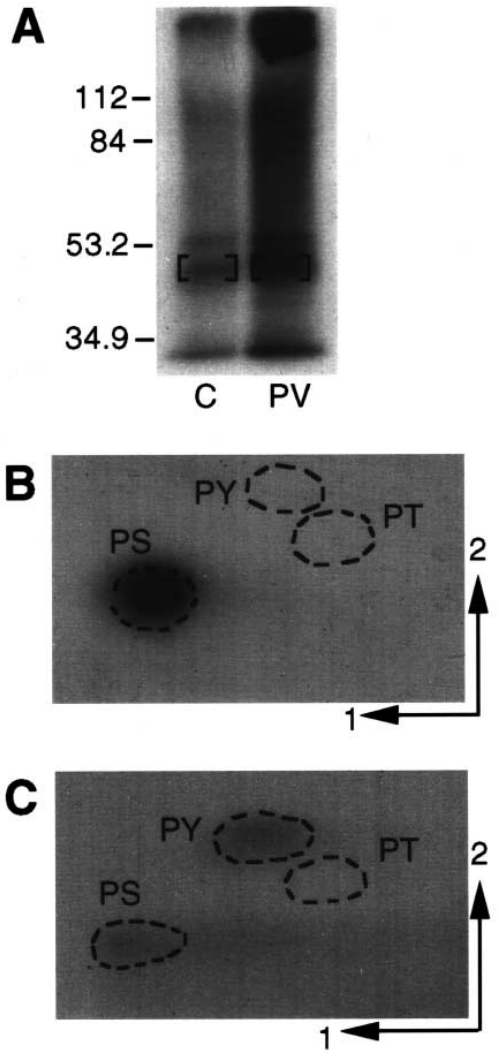

Fig. 3. Phosphoamino acid analysis of Cx43. Cells were labeled with ^{32}P for 4 h before immunoprecipitation of Cx43 and Western blotting. During the last 30 min, the cells were vehicle-treated or exposed to 30 μM pervanadate. A: The position of labeled Cx43 was identified by autoradiography, and the boxed areas of the corresponding blot were cut out and subjected to acid hydrolysis. C, control; PV, pervanadate. The positions of the prestained standards on the blot are marked on the left. The rectangular mark at the top of the PV lane is one of several position/identification markers for alignment of the blot and autoradiogram. The autoradiogram was exposed for 1 h. B, C: Separation of the phosphoamino acids was by (1) electrophoresis and (2) ascending chromatography. B: Phosphoamino acid analysis of Cx43 from control cells. C: Phosphoamino acid analysis from pervanadate-treated cells. The positions of phosphoamino acid standards are marked by the stippled lines (PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine). The autoradiograms in B and C were exposed for 15 days with intensifying screens at −70°C.

1, upper panel). The immunoprecipitation of Cx43 was not quantitative (not shown).

It was investigated if the compounds induced tyrosine phosphorylation of Cx43. Cx43 immunoprecipitates were probed with a combination of a phosphotyrosine-specific phosphatase from *Yersinia enterocolitica*, the anti-Cx43 antiserum (Fig. 2, lower panel) and a monoclonal antiphosphotyrosine antibody (Fig. 2, upper panel). Treatment of the immunoprecipitates with the *Yersinia* phosphatase gave no or very little change in the Cx43 band pattern from control cells and cells treated with 10 mM molybdate or 10 mM vanadate (Fig. 2, lower panel). Immunoprecipitates from cells exposed to H_2O_2 (3 mM), permolybdate (300 μM) and pervanadate (30 μM) showed clear changes subsequent to the *Yersinia* phosphatase treatment. The lower non-phosphorylated Cx43 band reappeared strongly together with bands corresponding to the two major upper bands in immunoprecipitates from untreated cells, although the upper bands reappeared with lower intensity than in the control. This could suggest a decrease in serine phosphorylation of Cx43.

The antiphosphotyrosine monoclonal antibody did not react against Cx43 immunoprecipitates from control cells and molybdate-treated cells, except for the weak non-specific reaction against the immunoglobulin heavy chain (Fig. 2, upper panel). The antibody reacted only very weakly against immunoprecipitates from cells treated with vanadate (10 mM). This reaction was eliminated by the treatment of the immunoprecipitates with the phosphotyrosine-specific phosphatase (Fig. 2, upper panel). The antiphosphotyrosine antibody reacted strongly against the Cx43 immunoprecipitates from cells treated with H_2O_2 (3 mM), permolybdate (300 μM) or pervanadate (30 μM) for 30 min (Fig. 2, upper panel). The region of antiphosphotyrosine reaction corresponded to a somewhat extended P1-P2 area, with no reaction in the NP area. The reactions were eliminated by the *Yersinia* phosphatase treatment (Fig. 2, upper panel).

Cx43 immunoprecipitates from control and pervanadate-treated cells were subjected to phosphoamino acid analysis (Fig. 3). Cx43 was immunoprecipitated from ^{32}P-labeled cells. The areas of the blot to use for acid hydrolysis were identified by autoradiography (Fig. 3A). In contrast to the relatively clear Cx43 P1/P2 bands obtained with horseradish peroxidase/chloronaphthol staining (Figs. 1 and 2, lower panels), the autoradiogram (Fig. 3A) of ^{32}P-labeled Cx43 could not distinguish between P1 and P2. This has also been observed previously [37]. Only phosphoserine could be detected in Cx43 from control cells (Fig. 3B), while both phosphoserine and phosphotyrosine were evident in Cx43 from pervanadate-treated cells (Fig. 3C). Consistent with the lower intensity of the P1/P2 bands in *Yersinia* phosphatase-treated Cx43 immunoprecipitates described above (Fig. 2), pervanadate caused an apparent decrease in the content of phosphoserine in Cx43 relative to the control. The acid hydrolysis appeared to be incomplete in some of the experiments (much radioactivity around the origin). We have therefore not quantified the decrease in phosphoserine content.

4. Discussion

Tyrosine phosphorylation of connexins in intact cells has only been found in cells transfected with the v-*src* [8,32,33] and v-*fps* oncogenes [34]. Both encode for activated tyrosine kinases. The activated epidermal growth factor receptor does not phosphorylate Cx43 directly [11], but rather through the activated mitogen-activated protein kinase [7,38]. However, the epidermal growth factor receptor tyrosine kinase may directly phosphorylate tyrosines in Cx32 in a cell-free system, but with a low efficiency [39]. We show here that pervanadate and permolybdate at μM concentrations and H_2O_2 at mM concentration cause tyrosine phosphorylation of Cx43 in in-

tact and normal cells. Per-compounds may therefore be useful tools to study the effects of tyrosine phosphorylation of Cx43.

Pervanadate [29] and permolybdate [31] have prominently higher biological potency than their parent compounds. The induction of phosphotyrosine in Cx43 by the present compounds can be achieved in two ways. They may exclusively inhibit one or more PTPases that act on Cx43, thereby disclosing a tyrosine kinase that has Cx43 as its normal substrate. Alternatively, they may indirectly or directly activate a tyrosine kinase that acts on Cx43. c-Src and c-Fps could be potential candidates as Cx43 tyrosine kinases based on the Cx43 phosphorylating activities of their activated viral homologues [8,32–34]. It was recently demonstrated that c-Src could phosphorylate tyrosines in Cx43 in a cell-free system [40]. Tyrosine phosphorylation of c-Src is involved in both positive and negative regulation of its activity [41]. There is no information if or how pervanadate and permolybdate affect the phosphorylation status of c-Src. Potentially, per-compounds could contribute to the activation of c-Src indirectly by generating numerous alternative binding sites for the c-Src SH2 domain due to the massive increase in cellular tyrosine phosphorylation. In support, the Src-family tyrosine kinases Fyn and Lck were activated by pervanadate in T-cells [23–25]. Members of other tyrosine kinase families [42,43] can be activated in cells by one or more of the present compounds, and might therefore also be considered as potential Cx43 tyrosine kinases.

The present results suggest that gap junctions in non-transformed cells might be targets for tyrosine kinases along with other types of cellular junctions and adhesion molecules [44–47]. The normal content of phosphotyrosine in Cx43 is clearly below the limit of detection. This could be achieved if a very small subpopulation of Cx43 is transiently tyrosine phosphorylated. Tyrosine phosphorylation may occur on Cx43 molecules incorporated into functional gap junctional channels, and thereby affect the open-state probability of the channels or other factors that affect their gating properties. However, since the Cx43 NP species disappears after exposure to the per-compounds, and it reappears after treatment of immunoprecipitates with a PTPase, it is clear that the treatments can induce tyrosine phosphorylation on the previously non-phosphorylated Cx43 species. The Cx43 NP species may not be incorporated into functional gap junctional channels in the cell membrane [37,48]. In this case, tyrosine phosphorylation may not immediately affect the gap junctional intercellular communication, but rather the Cx43 transport kinetics or the assembly into connexons. We are presently studying these alternatives.

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