receptors. The effect of serotonin was neither mimicked nor potentiated by dibutyryl cAMP or isobutyl-methylxanthine, consistent with a serotonin type 2 mediated effect. Omission of calcium from the incubation medium abolished both serotonin and A23187 induced increases in prostacyclin synthesis, suggesting the involvement of calcium ions in the transduction of this response.

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Calcium-dependent conversion of procollagen to collagen and its inhibition by other divalent cations

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Collagens, a family of closely-related, yet genetically distinct proteins, are synthesized as a precursor, procollagen, that contains noncollagenous extensions at both the N- and C-terminal ends of the molecule. These extensions are removed by two specific enzymes, procollagen N-proteinase and procollagen C-proteinase, respectively (for review on collagen, see Refs. 1–5). Previous studies [6, 7] had shown that the activity of procollagen N-proteinase is inhibited by EDTA, suggesting that the enzyme requires calcium or another divalent cation for its activity. Similarly, the activity of partially purified procollagen C-proteinase is inhibited by EDTA [8]. Our previous studies, employing a pulse-chase technique, had also indicated that the extracellular conversion of type II procollagen to collagen is inhibited by EDTA *in vitro*, and that the inhibition can be reversed by the addition of calcium [9]. In the present study, we

have examined the effects of several divalent cations on the conversion of type II procollagen to collagen *in vitro*.

Materials and methods

For pulse-chase experiments, 17-day-old chick embryo sterna were incubated in a medium containing 1.5 mM KCl, 120 mM NaCl, 4 mM $NaHCO_3$, 1.4 mM $MgCl_2 \cdot 6H_2O$, 13 mM dextrose, 50 μg/ml ascorbic acid, 50 μg/ml β-aminopropionitrile, 5% dialyzed fetal calf serum, and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) buffer, pH 7.5 [9, 10]. After a 30-min preincubation, [^{14}C]proline (NEC-280; sp. act. 280 mCi/mmole) was added, and the incubation was continued for 30 min at 37° (pulse). Further incorporation of radioactivity was then inhibited first by the addition of 500 μg/ml of [^{12}C]proline, followed in 5 min by the addition of 200 μg/ml of cycloheximide [10]. After a 5-min delay, the test compounds were added and the incubations were continued for 120 min, unless otherwise indicated. The radioactive proteins were then extracted with 3% sodium dodecyl sulfate (SDS) at 100°, in the presence of protease inhibitors [10]. The collagenous polypeptides were separated by SDS-polyacrylamide slab gel electrophoresis, using 6% gels, as indicated previously [10, 11]. The radioactive peptides were visualized by fluorography [12], and the bands were quantitated by scanning at 700 nm using an integrating densitometer (ADC-18, Gelman). The conversion of procollagen to collagen was integrated as:

$$\frac{100 \times \alpha}{\text{pro}\alpha + \text{pC}\alpha + \text{pN}\alpha + \alpha} (\%).$$

The relative conversion in the test samples was expressed as a percentage of the corresponding control.

Results

Chick embryo sterna were labeled with [^{14}C]proline for 30 min (pulse), and the incorporation of radioactivity into protein was inhibited by the addition of unlabeled proline and cycloheximide (chase). As reported previously [9], the predominant collagenous polypeptide at the end of the pulse was proα (II), when the radioactive proteins were examined by SDS-polyacrylamide slab gel electrophoresis (Fig. 1, lanes A and B). Some pCα chains were also present, indicating that a partial conversion of procollagen to pC-collagen occurred during the pulse. If the sterna were further incubated in the original medium devoid of calcium for 120 min following inhibition of the protein synthesis, little if any additional conversion took place, and no α-chains were present at the end of the chase (Fig. 1, lanes C and D). However, if 0.5 mM Ca^{2+} was added to the incubation medium at the beginning of the chase period, most the precursor polypeptides were efficiently converted to α-chains (Fig. 1, lanes E and F). In similar experiments, several other divalent cations were tested in the pulse-chase system described above. The results indicated that the addition of 1 mM Zn^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, or Cu^{2+}, in the absence of calcium, did not catalyze the conversion (data not shown). Thus, these cations were unable to substitute for Ca^{2+} in the conversion reactions.

In further studies, 1 mM Ca^{2+} was added to the incubation medium at the end of the labeling period, immediately followed by the addition of one of the divalent cations indicated above; the incubation was then allowed to continue for an additional 120 min. The results indicated that 1 mM Zn^{2+}, in the presence of 1 mM Ca^{2+}, completely inhibited the conversion, and no α-chains were detectable by SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes G and H). Similar results were observed with 1 mM Hg^{2+} and Cu^{2+}, while 1 mM Mn^{2+}, Ni^{2+}, Pb^{2+} and Co^{2+} were not effective in inhibiting the Ca^{2+}-dependent conversion. Testing of Zn^{2+} in various concentrations, in the presence of 0.1, 1.0 and 10.0 mM Ca^{2+}, indicated the Zn^{2+} inhibited 50% of the conversion in a $Zn^{2+}:Ca^{2+}$ molar ratio of 1:9.3 (Fig. 2).

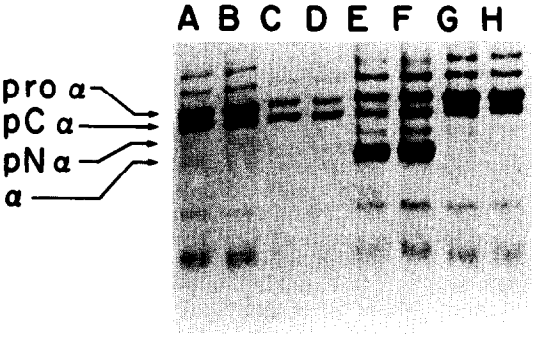

Fig. 1. Conversion of type II procollagen to collagen *in vitro*. Sterna from 17-day-old chick embryos were pulse-labeled with [^{14}C]proline for 30 min, and the incorporation of radioactivity into protein was inhibited by the addition of [^{12}C]proline and cycloheximide, as indicated in Materials and Methods. The test substances were then added, and the incubations were continued for 120 min. The samples were examined by SDS-polyacrylamide gel electrophoresis followed by fluorography. Lanes A and B: samples at the end of the pulse-labeling period; lanes C and D: samples after 120 min chase in medium without Ca^{2+}; lanes E and F: samples after chase in medium containing 0.5 mM Ca^{2+}; and lanes G and H: samples after chase in medium containing 1 mM Zn^{2+} and 1 mM Ca^{2+}. The migration positions of proα-, pCα-, pNα- and α-chains of type II procollagen and collagen are indicated in the figure.

Further experiments were designed to test the reversibility of the Zn^{2+} or Cu^{2+} inhibition by first incubating sterna for 120 min in the medium containing these cations in 0.5 or 1.0 mM concentrations, respectively, and then replacing the medium with fresh medium containing 5 mM Ca^{2+}. The results indicated that, during the subsequent 120-min incubation, the inhibitions by Zn^{2+} or Cu^{2+} could not be reversed by excess Ca^{2+}. Also, brief chelation of Zn^{2+} or Cu^{2+} by 10 mM Na_2 EDTA, followed by replacement of medium with fresh medium containing 5 mM Ca^{2+}, did not reverse the inhibition (data not shown).

Discussion

The conversion of type II procollagen to collagen involves enzymatic removal of the N- and C-terminal extensions from the molecule [9]. Previous observations (see Refs. 1–5) have suggested that two separate enzymes catalyze the removal of these extensions, but specific N- and C-terminal proteinases have not been isolated from tissues synthesizing type II collagen. However, partially purified N- and C-proteinases, isolated from chick embryo tendons and calvaria, tissues synthesizing type I collagen, have been shown to cleave the extensions on type II procollagen, in addition to type I procollagen [8, 13]. Both these enzymes were inhibited by EDTA and other metal chelators, suggesting a requirement for divalent cations [7, 8]. The activity of N-proteinase could be partially restored by the addition of Ca^{2+}, but Mg^{2+} and Mn^{2+} were equally effective [7]. In our study, several divalent cations, including Mn^{2+}, were unable to substitute for Ca^{2+} in equimolar concentrations. Thus, at this point it is unclear whether the enzyme catalyzing the removal of the N-terminal extension from type II procollagen in cartilage is different from the enzyme purified from chick embryo tendons.

Previously, the activity of N-proteinase purified from

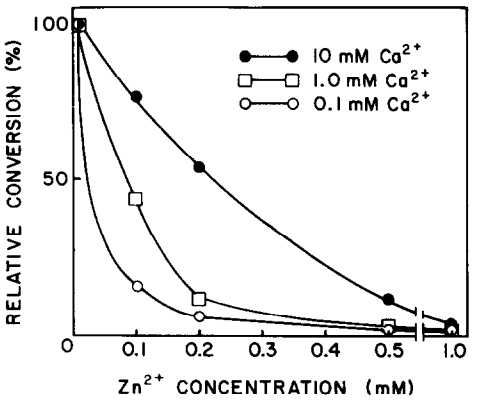

Fig. 2. Inhibition of Ca^{2+}-dependent conversion of type II procollagen to collagen by Zn^{2+}. Sterna were pulse-labeled for 30 min, as indicated in Fig. 1 and Materials and Methods. Ca^{2+}, either in 10.0, 1.0, or 0.1 mM concentrations, together with various concentrations of Zn^{2+} was added, and the incubations were continued for 120 min. The samples were examined by SDS-polyacrylamide gel electrophoresis; the ^{14}C-polypeptides were visualized by fluorography and quantitated by densitometry (see Materials and Methods). The conversion of type II proα-chains to α-chains is expressed as a percentage of the control containing no Zn^{2+} at each given Ca^{2+} concentration. The Zn^{2+} concentrations eliciting 50% inhibition of the conversion, extrapolated from the inhibition curves, are 20, 85 and 225 μM in the presence of 0.1, 1.0 and 10.0 mM Ca^{2+} respectively.

chick embryo tendons was shown to be inhibited by Zn^{2+}, Co^{2+}, Fe^{2+} and Pb^{2+}, in the presence of Ca^{2+} [7]. In our study, Zn^{2+}, Cu^{2+}, and Hg^{2+} were potent inhibitors of the Ca^{2+}-dependent conversion of type II procollagen to collagen, but Mn^{2+}, Ni^{2+}, Pb^{2+} and Co^{2+} had no effect in the presence of calcium in equimolar concentrations.

Detailed studies on the metal requirements for the activity of C-proteinase isolated from chick embryo calvaria have not been presented. It appears, however, that the enzyme purified by Njieha *et al.* [8] is different from that in chick embryo sterna, since the calvaria enzyme is completely inhibited by 5% fetal calf serum, while the conversion of procollagen to collagen during incubations of sterna was unaffected by the presence of 5% fetal calf serum. Also, the enzyme purified from calvaria is only minimally inhibited by 50 mM L-arginine [8], while the conversion of type II pC-collagen to collagen in sterna [10], as well as the removal of the C-terminal extension from type I collagen synthesized by chick embryo tendons [14], are effectively inhibited by L-arginine in this concentration.

Previous studies [15] have demonstrated that Zn^{2+} is a potent inhibitor of prolyl and lysyl hydroxylation during the intracellular biosynthesis of type I procollagen, and that incubation of matrix-free chick embryo tendon cells with 10 μM Zn^{2+} can cause an intracellular accumulation of the collagenous polypeptides. In our study, the test compounds were added to the incubation medium after the inhibition of protein synthesis at the end of the pulse-labeling period, a total of 40 min having elapsed from the initiation of the pulse. Since the average secretion time of a newly synthesized type II procollagen is about 28 min [16, 17], most of the [^{14}C]procollagen molecules had been secreted into the extracellular space at the time of the addition of the test compounds. Thus, the inhibition of conversion noted with Zn^{2+} and other divalent cations appeared to be independent of the intracellular events and was probably mediated through direct inhibition of the enzymes catalyzing the extracellular conversion reactions. In support of this suggestion is the previous demonstration [18] that the activity of an N-proteinase for type III procollagen, purified from cultures of calf tendon fibroblasts, is inhibited by Zn^{2+} and Cu^{2+}.

In summary, our results indicate that the removal of N- and C-terminal extensions from type II procollagen specifically required Ca^{2+}, but that the conversion was inhibited by Zn^{2+}, Cu^{2+}, and Hg^{2+}. The inhibition by Zn^{2+} or Cu^{2+} was not reversed by the excess Ca^{2+}, suggesting a tight binding of these cations in the inhibitory site. The results thus suggest that the conversion of procollagen may be modulated by changes in the tissue concentrations of various cations.

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