Purification and characterization of calcium-calmodulin kinase II from human parathyroid glands

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ABSTRACT
Calmodulin has been identified in parathyroid cells and is thought to play an important role in the production or secretion of parathyroid hormone. However, a detailed investigation of calmodulin-binding proteins in parathyroid glands has not been conducted. In this study, we attempted to determine the presence of calmodulin-binding protein in human parathyroid adenoma by affinity chromatography. The eluted protein from a calmodulin-coupled Sepharose 4B column with EGTA was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis which revealed a major protein band of $M_r$ 50,000. A $Ca^{2+}$/calmodulin-dependent protein kinase activity was detected at the protein peak using dephosphorylated casein as a substrate. The 50 kDa band was identified as calcium/calmodulin-dependent protein kinase II (CaM-kinase II) by immunoblotting. The substrate specificity, pH dependency and affinity for calmodulin of this enzyme were identical to those of CaM-kinase II from rat brain. Also, the kinase activity was sensitive to KN-62, a specific inhibitor of CaM-kinase II. In total, 0.48 mg of this kinase was purified from 3 g human parathyroid adenoma.

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INTRODUCTION
Parathyroid hormone (PTH) is the most important and effective hormone regulator of the plasma concentration of $Ca^{2+}$, and parathyroid function is regulated primarily by the extracellular calcium concentration (Habener, Rosenblatt & Potts, 1984). Various studies (Shoback, Thatcher, Leombruno & Brown, 1984; Nemeth, Wallace & Scarpa, 1986; Nygren, Gylfe, Larsson et al. 1988) have suggested that the unique $Ca^{2+}$ sensor mechanism of parathyroid cells involves cation-activated $Ca^{2+}$ permeability of the plasma membrane, which causes a two- to threefold increase in the intracellular concentration of $Ca^{2+}$ by only small increases in the extracellular concentration of $Ca^{2+}$ within a very narrow range. There should, therefore, be a messenger system which transmits the increase of intracellular concentration of $Ca^{2+}$ to secretion of PTH.

Calmodulin is one of the well-known $Ca^{2+}$-binding proteins and acts as an intracellular calcium receptor modulating the function of a variety of enzymes which regulate intracellular cyclic AMP or calcium-signalling systems (Hidaka & Hartshorne, 1985). Brown, Dawson-Hughes, Wilson & Adragna (1981) identified calmodulin in dispersed bovine parathyroid cell preparations and pathological human parathyroid glands. In that study, cells from secondary hyperparathyroidism had significantly greater levels of calmodulin than either normal cells or adenomas, but they could not define its pathophysiological significance nor the role of calmodulin in PTH secretion. In the present study, we investigated the presence of calmodulin-binding proteins in parathyroid glands using a calmodulin-bound Sepharose 4B column; calcium/calmodulin-dependent protein kinase II (CaM-kinase II) was identified.

MATERIALS AND METHODS
NK-62 (Tokumitsu, Chijiwa, Hagiwara et al. 1990), H-7 (Hidaka, Inagaki, Kawamoto & Sasaki, 1984) and W-7 (Hidaka, Asano, Iwadare et al. 1978) were synthesized as described previously. Calmodulin was purified from bovine brain (Endo, Tanaka, Isobe et al. 1981). A polyclonal antibody to CaM-kinase II was produced by injecting a synthetic peptide, correspond-
Calmodulin-binding proteins were eluted with buffer B which contained 2 mmol EGTA/l. The absorbance at 280 nm showed a marked peak after fraction 36 and SDS-PAGE of eluted fractions displayed a major 50 kDa band (Fig. 1a). 

Calmodulin-dependent kinase activities were detected by the enzyme peak using dephosphorylated casein as a substrate (Fig. 2), and this 50 kDa band was immunopositive with the polyclonal antibody to CaM-kinase II by the immunoblotting method (Fig. 1b). This 50 kDa band was also immunoprecipitated to the gel fraction and no remaining bands were seen in the supernatant fraction (data not shown).

It has already been confirmed that the CaM-kinase II polyclonal antibody used in this study reacts with the α and β subunit of rat brain (Tokumitsu et al. 1990), and this enzyme was supposed to contain only the M_cal 50 000 subunit, which is characteristic of the CaM-kinase II from non-neuronal organs (Fukunaga, Goto & Miyamoto, 1988).

The purification procedure of CaM-kinase II from human parathyroid adenoma is summarized in Table 1. Specific activity rose to 1400-fold and total activity was also increased up to about fivefold, so the recovery ratio exceeded 100%. This could be explained by the co-existence of an unknown inhibitory substance in the precolumn chromatography fractions. In total, 0.48 mg CaM-kinase II was purified from 3 g human parathyroid adenoma.

Characterization of enzyme

Effect of calmodulin concentration

The concentration/velocity responses of the purified enzyme are shown in Fig. 3. The assay condition chosen, 0.1 μmol calmodulin/l, sufficed for the activation of this enzyme. Lineweaver–Burk plotting linearized this curve and gave an associated constant (K_a) of 240 nmol for calmodulin.
FIGURE 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting of eluted fractions from a calmodulin affinity column. (a) Electrophoresis was carried out in 10% polyacrylamide slab gels and 50 kDa homogenous bands appeared from fraction numbers 38 to 46. (b) These 50 kDa bands were immunopositive with the polyclonal antibody to the calmodulin-kinase II.
Fraction number figure 2. Elution profile of calmodulin affinity chromatography. The flow rate was 10 ml/h and each fraction was collected into a 1-15 ml tube. From fraction no. 31 (indicated by the arrow), the buffer was changed to buffer B (see text), and the bound protein was eluted from fraction no. 36. Fractions were assayed for protein kinase activity in the absence (□) and presence (■) of Ca\textsuperscript{2+}/calmodulin. The absorbance at 280 nm (○) is also indicated.

### TABLE 1. Purification of calmodulin-kinase II from human parathyroid gland

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (pmol P/min)</th>
<th>Specific activity (pmol P/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>18</td>
<td>135</td>
<td>4114</td>
<td>30.5</td>
</tr>
<tr>
<td>Supernatant</td>
<td>15</td>
<td>100</td>
<td>7285</td>
<td>72.9</td>
</tr>
<tr>
<td>Elution from calmodulin affinity on Sepharose 4B</td>
<td>11</td>
<td>0.48</td>
<td>20 010</td>
<td>41 687.5</td>
</tr>
</tbody>
</table>

**Substrate specificity**

In order to facilitate comparison of the enzyme with the CaM-kinase II from rat brain, the ability to phosphorylate five substrates frequently used for assaying protein kinases was examined. As shown in Table 2, these two enzymes have almost the same specificity for these substrates. For comparison, the rate of phosphorylation was calculated as pmol \(^{32}\)P/min per mg substrate, and the per cent ratio to \(^{32}\)P incorporation to synapsin I was determined. Synapsin I was the best substrate for both kinases.

**Effect of pH on kinase activity**

Figure 4 shows the effect of pH on casein phosphorylation of the parathyroid kinase. The optimal pH was around 6.5, with the second optimal peak around pH 8.5. These data are identical with those for CaM-kinase II from the rat brain (Yamauchi & Fujisawa, 1984).

**Effects of various inhibitors on kinase activity**

Figure 5 shows the effects of three specific inhibitors on parathyroid kinase activity. More than 60% of the kinase activity was inhibited by adding 10 \(\mu\)mol KN-62/l, which is a specific inhibitor of CaM-kinase II. The median inhibitory concentration (IC\(_{50}\)) was estimated as 2 \(\mu\)mol/l. W-7, a specific inhibitor of calmodulin, also weakly inhibited the activity and the IC\(_{50}\) was approximately 18 \(\mu\)mol/l. H-7, a specific inhibitor of protein kinase C, had no effect on this kinase activity.

**Intrinsic substrate of parathyroid CaM-kinase II**

Parathyroid tissue was homogenized with 10 vol. buffer C and centrifuged at 100 000 g for 30 min. The supernatant was boiled at 90 °C for 1 min to eliminate other
kinase activities, and protein phosphorylation reactions were performed. The proteins phosphorylated by CaM-kinase II were then identified by autoradiography. Figure 6 shows one major phosphorylated band at 50 kDa and three other weakly phosphorylated bands at 58, 63 and 74 kDa in the supernatant. They were phosphorylated in a Ca^{2+}-dependent manner, and were also inhibited by KN-62.

**DISCUSSION**

CaM-kinase II is known to have relatively broad substrate specificity, and its physiological role is postulated to prolong the effects triggered by transient increases in intracellular Ca^{2+} signals (Miller & Kennedy, 1986; Lai, Nairn & Greengard, 1987). In the nervous system, it is thought to play a role in long-term modulation of synaptic transmission (Browning, Hauganir & Greengard, 1985; Colbran, Schworer, Hashimoto et al. 1989) and, in the adrenal medulla, it is thought to phosphorylate tryosine hydroxylase and regulate catecholamine secretion (Yamauchi, Nakata
used calmodulin-affinity chromatography but could not obtain detectable levels of protein.

We initially intended to determine the presence of calmodulin-binding proteins within the parathyroid gland to investigate the intracellular Ca$^{2+}$ signal transduction system within parathyroid cells, which should play the critical role in the regulation of PTH secretion. Using a single step of calmodulin-affinity chromatography, we could purify CaM-kinase II. From our data, 0.36% of the total parathyroid protein or 0.016% of total parathyroid weight consisted of CaM-kinase II. This amount is virtually the same as that in the brain (Bennett, Erondu & Kennedy, 1983). We also revealed that CaM-kinase II from parathyroid tissue is similar to that from brain, except that it consists of only an α subunit. The fact that CaM-kinase II was the most abundant calmodulin-binding protein suggests that CaM-kinase II plays an important role in the Ca$^{2+}$-signal transduction system in parathyroid cells.

Baty, Rouse & Hamilton (1988) could not detect the Ca$^{2+}$/calmodulin-dependent kinase activity in bovine parathyroid extract and speculated that this might be due to the tissue employed or some methodological differences. In the present study, CaM-kinase II activity could be eluted from human and bovine parathyroid glands (data not shown). We suggest that the failure of Baty et al. (1988) to detect CaM-kinase II was due to the differences in assay conditions, because histone H1 which they used as the substrate is not a good substrate for CaM-kinase II. In our study, little or no incorporation of $^{32}\text{P}$ into histone H1 was seen.

When we used fresh parathyroid tissue extract as substrate for CaM-kinase II to investigate the intrinsic substrate, numerous proteins were phosphorylated even in the presence of 10 mmol EGTA/l, suggesting contamination by other Ca$^{2+}$/calmodulin-independent protein kinases. These Ca$^{2+}$/calmodulin-independent phosphorylations were eliminated by boiling the extract. $^{32}\text{P}$Orthophosphate labelling of primary cultured human parathyroid adenoma cells also revealed a 50 kDa phosphorylated protein (data not shown). Kinder et al. (1987) also noticed this phosphorylated band and suggested that it would be the autophosphorylated α subunit of CaM-kinase II. We have performed the immunoprecipitation study using CaM-kinase II antibody which could bind the autophosphorylated form of the enzyme as well as the non-phosphorylated one (Tokumitsu et al. 1990). The purified kinase II band (50 kDa) was precipitated on the SDS-PAGE gel but, by autoradiography, the 50 kDa phosphorylated band was not precipitated (data not shown), suggesting the existence of another substrate protein different from the autophosphorylated CaM-kinase II α subunit.

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FIGURE 6. Intrinsic substrate of calmodulin-kinase II in the parathyroid tissue. Each lane contains 20 μl parathyroid tissue extract and 0.4 μg purified enzyme. Lane 1, instead of CaCl$_2$, 10 mmol EGTA/l was added to the assay mixture. Lane 2, in the presence of 1 mmol Ca$^{2+}$/l, but absence of calmodulin. Lane 3, in the presence of 1 mmol Ca$^{2+}$/l and 0.1 μmol calmodulin/l. Lane 4, 10 μmol KN-62/l was added. The incubations were carried out for 1 min at 30°C and terminated by adding SDS sample buffer containing 8 mol urea/l.

& Fujisawa, 1981; Campbell, Hardie & Vulliet, 1986). Kinder, Delahunt, Jamieson & Gorelick (1987) identified Ca$^{2+}$/calmodulin-dependent protein kinase activity derived from human hyperplastic parathyroid tissue. They used gel filtration and demonstrated kinase activity at the fractions of molecular weight of approximately $5.5 \times 10^5$. Kinder et al. (1987) investigated the endogenous parathyroid phosphoproteins by autoradiography and found a Ca$^{2+}$/calmodulin-independent 49 kDa phosphorylated band. They also
Previous measurement of intracellular Ca$^{2+}$ in parathyroid cells using fura-2 indicated that high extracellular Ca$^{2+}$ initially produces a transient spike-like increase in intracellular Ca$^{2+}$ within a few seconds, followed by a sustained increase in intracellular Ca$^{2+}$ lasting several minutes (Nemeth & Scarpa, 1986). Divalent cations such as Mg$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$ evoke the spike-like increase in intracellular Ca$^{2+}$, but not the sustained response (Nemeth & Scarpa, 1987). Like Ca$^{2+}$, however, the divalent cations inhibit PTH release (Nemeth & Scarpa, 1987). Phorbol myristate acetate also diminishes this initial spike-like increase in intracellular Ca$^{2+}$ and affects PTH release (Muff & Fisher, 1986; Membreno, Chen, Woodley et al. 1989). These phenomena suggest that the initial Ca$^{2+}$ spike is critical for the regulation of PTH release. Thus, the intracellular Ca$^{2+}$-calmodulin messenger system should have an important role in regulating PTH secretion from parathyroid cells through the activation of CaM-kinase II. Since the parathyroid CaM-kinase II we found was sensitive to KN-62, a synthetic inhibitor for CaM-kinase II (Tokumitsu et al., 1990), it would be fruitful to investigate the influence of such a compound on PTH secretion from intact parathyroid cells.

REFERENCES


