

## Characterization of $\gamma$ - and $\delta$ -subunits of $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in rat gastric mucosal cell populations

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We searched for the occurrence of a  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase in rat gastric cell types as a likely member in the chain of gastrin- and muscarinic-receptor-mediated signal transmission. A  $\text{Ca}^{2+}$ - and calmodulin-dependent phosphorylation of major 50, 60 and 100 kDa substrates was observed in parietal cell cytosol and a major 60 and 61 kDa protein doublet was found to bind  $^{125}\text{I}$ -calmodulin in  $^{125}\text{I}$ -calmodulin-gel overlays. A specific substrate of the multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II, autocamtide II, was phosphorylated in a

calmodulin-dependent manner. The specific inhibitor of this enzyme, KN-62, antagonized protein kinase activity. RNA extracted from gastric mucosal cells was shown to contain sequences of the  $\gamma$ - and  $\delta$ - but not  $\alpha$ - and  $\beta$ -subunits of the calmodulin-dependent kinase II, and mRNA of both subtypes was demonstrated in highly purified parietal, chief and mucous cells. A calmodulin-dependent kinase II composed of  $\gamma$ - and  $\delta$ -subunits is a likely mediator of  $\text{Ca}^{2+}$ -dependent signal transmission in these populations of gastric cells.

### INTRODUCTION

Muscarinic-receptor-mediated stimulation of acid, pepsinogen and mucous secretion by rat gastric cells is thought to involve the phosphoinositide second messenger system (Soll, 1981; Chew and Brown, 1986; Pfeiffer et al., 1986, 1988; Puurunen and Schwabe, 1987; Seidler and Pfeiffer, 1991). In this system, receptor-mediated activation of phospholipase C results in the cleavage of phosphatidylinositol 4,5-trisphosphate into inositol trisphosphate and diacylglycerol (Berridge, 1987; Nishizuka, 1988). Inositol trisphosphate elevates intracellular  $\text{Ca}^{2+}$  and thereby activates  $\text{Ca}^{2+}$ -dependent processes (Yamamoto et al., 1985; Nishimura et al., 1988). Among these, the activation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaM kinase II) appears to be involved in gastric acid secretion, as inhibition of this kinase by the specific antagonist KN-62 (Tokumitsu et al., 1990) blocks acid secretion by isolated rabbit gastric parietal cells (Tsunoda et al., 1991).

The  $\text{Ca}^{2+}$ - and calmodulin-dependent kinases constitute a large family comprising substrate-specific kinases such as phosphorylase kinase from liver, myosin light chain kinase from numerous tissues, a type-III kinase phosphorylating a 100 kDa substrate observed in numerous tissues and a neuronal type-IV or GR-kinase (Nairn et al., 1985; Roush et al., 1988; Kameshita and Fujisawa, 1991; Nygard et al., 1991; Miyano et al., 1992). The multifunctional CaM kinase II phosphorylates a variety of substrates. At present, four different subtypes of this enzyme have been identified in rat brain, termed  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  (Kuret and Schulman, 1984; McGuinness et al., 1985; Bennet and Kennedy, 1987; Lin et al., 1987; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). The holoenzyme in rat brain has a molecular mass of 500–550 kDa and consists of multiple subunits primarily of the  $\alpha$  and  $\beta$  type (Schulman and Lou, 1989; Colbran et al., 1989). The single subunits are catalytically active and have molecular masses of 50–54 kDa for the  $\alpha$ -subunit and 60–62 kDa for the  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits.

In rabbit gastric parietal cells, the occurrence of CaM kinase II has been suggested (Ottsdottir et al., 1987) on the basis of phosphorylation of its typical 100 kDa substrate. Using a polyclonal antibody to brain CaM kinase II, Fukunaga et al. (1990) showed a positive reaction by immunohistochemistry in rat gastric glands. Two further studies showed a CaM kinase II-like phosphorylating activity in rabbit parietal cells (Tsunoda et al., 1991; Funasaka et al., 1992).

The present work reports some characteristics of a  $\text{Ca}^{2+}$ - and calmodulin-dependent kinase present in various rat gastric mucosal cell types. The data suggest that a CaM kinase II occurs in rat gastric mucosal cells. The subtypes present were identified by amplification of cellular mRNA after reverse transcription and are shown to be of the  $\gamma$  and  $\delta$  types. The CaM kinase II subtypes are shown to occur in all gastric cell types after purification of parietal, mucous and pepsinogen-producing cells to homogeneity.

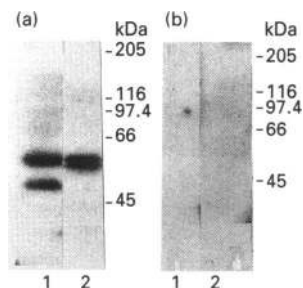
### EXPERIMENTAL

#### Animals and materials

Male Wistar rats weighing 200–250 g were obtained from a local breeding company. Chemicals were from Merck (Darmstadt, Germany) except when noted otherwise. Calmodulin, Percoll and phenyl-Sepharose were from Pharmacia/LKB (Bromma, Sweden). Affi-T-Thiosepharose was from Biomol, DE-52 from Whatman, KN-62 was synthesized by H. Hidaka, calmidazolium was from Boehringer-Mannheim and [ $\gamma$ - $^{32}\text{P}$ ]ATP was from NEN.

#### Enzyme preparation

Rats were anaesthetized with diethyl ether and killed by cervical dislocation. The stomach was removed, everted and filled with Pronase to produce single mucosal cells as described (Pfeiffer et al., 1988). Cells were homogenized in ice-cold homogenization buffer using a Polytron. The buffer consisted of 20 mM Tris/HCl,



**Figure 1** Autoradiograph of SDS/polyacrylamide gel of calmodulin overlay

Protein from rat brain cytosol (lane 1) or rat gastric parietal cells (lane 2) was separated on SDS/polyacrylamide gels and labelled with  $^{125}\text{I}$ -calmodulin in the presence of 1 mM  $\text{Ca}^{2+}$  (a) or 1 mM EGTA (b) after extensive washing as described in the Experimental section. The labelled bands in the rat brain had molecular masses of 50 and 60 kDa. Gastric cell supernatant shows a doublet at 60 and 61 kDa.

pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethanesulphonyl fluoride, 1 mM dithiothreitol, 1 or 5 mg/l leupeptin and 1 or 5 mg/l pepstatin. The homogenate was centrifuged (14000 g) for 15 min at 4 °C and the supernatant was used for further studies.

#### Assay of kinase activity

CaM kinase II assays were performed using autocamtide II [KKALRRQETVDAL (Hanson et al., 1989)] as substrate in a 50  $\mu\text{l}$  volume containing 10  $\mu\text{l}$  of enzyme preparation (3–15  $\mu\text{g}$  of protein), 50 mM Tris/HCl, pH 7.5, 20  $\mu\text{M}$  ATP, 10 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , 0, 0.1 or 1  $\mu\text{M}$  calmodulin, 1 mg/ml BSA, 0.2–2  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /assay and 20  $\mu\text{M}$  autocamtide II. Controls contained  $\text{Ca}^{2+}$  but no calmodulin. A variable degree of phosphorylation was obtained in the presence of  $\text{Ca}^{2+}$  but absence

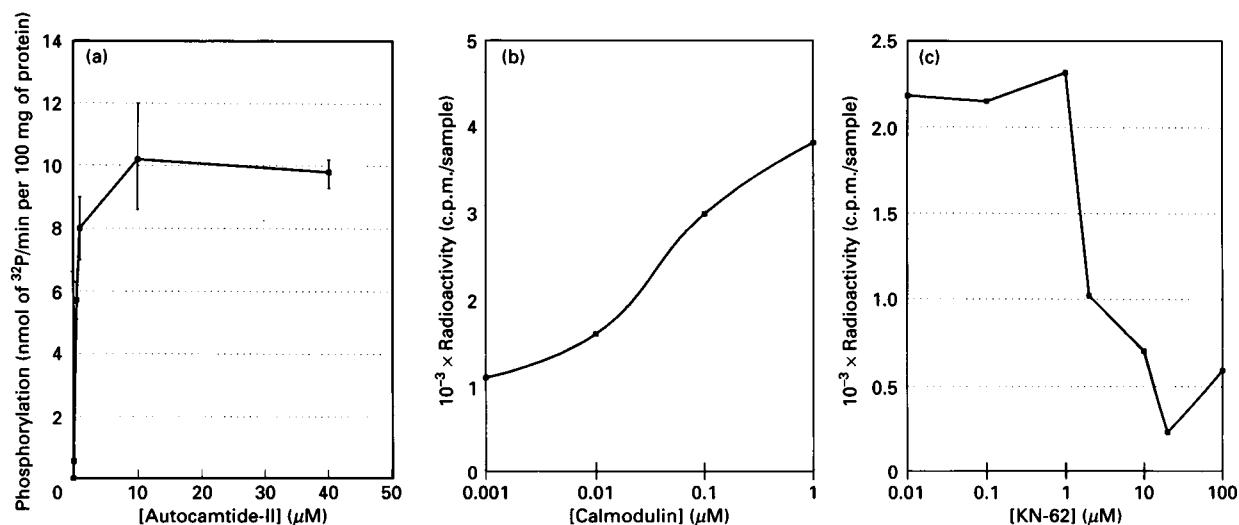
of calmodulin when 1 mM EGTA was used for determination of background. Enzyme activity in crude cytosol which was apparently calmodulin-independent was found to be partly but variably inhibited by KN-62, which suggests that endogenous calmodulin accounted for some of the 'calmodulin-independent' phosphorylation. However, by determining background in the presence of  $\text{Ca}^{2+}$ , only calmodulin-dependent activity was assayed and interference by  $\text{Ca}^{2+}$ -activated calmodulin-independent kinases was eliminated. The assay was started by addition of either enzyme preparation or  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . The reaction was linear over at least 5 min at 21 °C. After 3 min, the reaction was stopped with 25  $\mu\text{l}$  of 20% (w/v) trichloroacetic acid and a sample was applied to Whatman P81 phosphocellulose paper which was washed as described by Roskowski (1985) and dried before determination of radioactivity by Cerenkov counting in a Beckmann LS 6000 counter.

#### Phosphorylation of endogenous proteins

Parietal cell preparations were homogenized in the buffer described above, centrifuged for 15 min at 14000 g at 4 °C and incubated with 2–5  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ /assay for 0.5–4 min. The samples contained 2 mM EGTA, or 100  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , or 100  $\mu\text{M}$  free  $\text{Ca}^{2+}$  and 1  $\mu\text{M}$  calmidazolium (Boehringer-Mannheim) as a calmodulin antagonist. The free  $\text{Ca}^{2+}$  concentration was calculated with the program 'CALCON', kindly provided by Dr. J. S. Tash, Department of Cell Biology, Houston, TX, U.S.A. The reaction was stopped by the addition of SDS sample buffer, boiled for 2 min and separated by SDS/PAGE (7.5–15% gradient gels) by the method of Laemmli (1970) followed by autoradiography of the dried and stained gels to visualize labelled proteins.

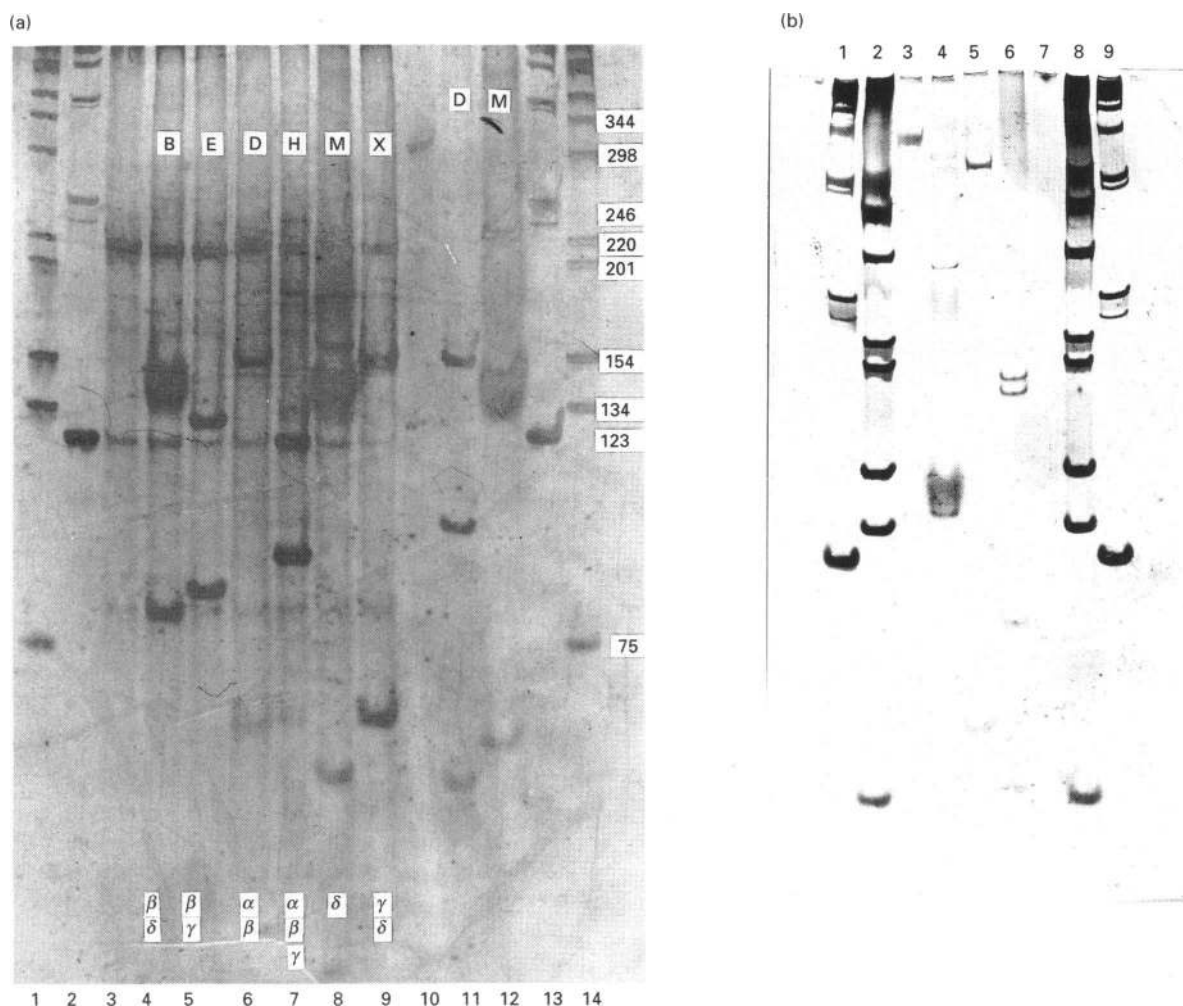
#### $^{125}\text{I}$ -calmodulin overlay

$^{125}\text{I}$ -calmodulin overlays were performed as described by Slaughter and Means (1987). Cytoplasmic proteins were sepa-



**Figure 2** Phosphorylation of autocamtide II in parietal cell cytosol

(a) Substrate-saturation curve. The curve shows the calmodulin-dependent phosphorylation obtained by subtracting values obtained in the presence of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  but without 100 nM calmodulin from those in the presence of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  plus 100 nM calmodulin. The data are means  $\pm$  S.D. from four independent experiments. (b) Calmodulin-dependence of phosphorylation. One representative experiment is shown which was repeated twice with similar results. (c) Inhibition of autocamtide II phosphorylation by KN-62. One representative experiment is shown which was repeated twice with similar results.



**Figure 3** Amplification of cDNA corresponding to the  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits from CaM kinase II in rat brain

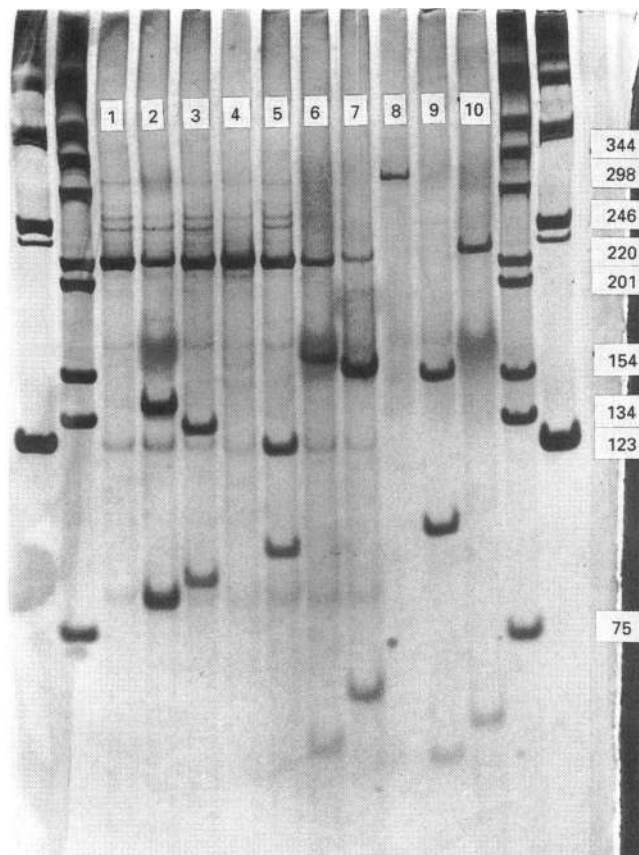
(a) The cDNA was amplified with either a pair of primers yielding a 219 bp sequence with  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits of CaM kinase II (lanes 3–9) or with primers specific for the  $\delta$ -subunit yielding a 309 bp sequence (lanes 10–12). Lanes 1 and 2 and 13 and 14 show markers with the length (bp) indicated on the right. After amplification of the cDNA as described in the Experimental section, the unpurified DNA was cut with the restriction enzymes indicated at the top. Lane 3, none; B, *Bst*XI ( $\beta$  and  $\delta$  140/79) [*Bst*XI added alone yielded a broad protein band around 140 bp as shown in Figure 5(b) (lane 15)], E, *Eco*RV ( $\beta$  and  $\gamma$  132/87); D, *Dra*II ( $\alpha$  156/63,  $\beta$  185/34); H, *Hae*III ( $\alpha$  125/94,  $\beta$  120,67,32,  $\gamma$  125/94); M, *Mae*III ( $\delta$  162/57); X, *Xho*II ( $\gamma$  and  $\delta$  156/63). At the bottom is indicated which CaM kinase subtype is cut by the enzymes. (b) The cDNA was amplified with a  $\beta$ -specific primer as detailed in the Experimental section yielding a 491 bp sequence. The outer two lanes contain the same standards as in (a). Lane 3, no restriction enzymes added; lane 4, *Bst*XI yielding restriction fragments of 283/140/68 bp; lane 5, *Eco*RV, 404/87 bp; lane 6, *Dra*II, 197/185/109 bp; lane 7, blank (all components except for RNA added to PCR); lanes 1 and 9, 123 ladder (Gibco); lanes 2 and 8, 1 kb ladder (Gibco).

rated on SDS/7–14% polyacrylamide gradient gels. <sup>125</sup>I-calmodulin was labelled using the Bolton–Hunter technique with reagents from Amersham (Dreieich, Germany). The gel was fixed, washed to remove SDS, and incubated with <sup>125</sup>I-calmodulin. After another extensive washing procedure, protein bands in the dried gel were visualized by autoradiography using Quanta III amplification screens (DuPont) at  $-70^{\circ}\text{C}$ .

#### Cell isolation

Cells were prepared to yield single-cell preparations as described above and in Pfeiffer et al. (1988). The isolated cells were then loaded into a Beckmann elutriator rotor at 1800 rev./min and eluted in six fractions with flow rates of 7, 14, 24, 30, 50 and 80 ml/min. The cells of the second, third and fifth fraction were layered on to preformed continuous Percoll gradients (60% Percoll) which had been preformed by spinning for 20 min at

30000 g. The samples were spun for 20 min at 2400 g (3000 rev./min). The second elutriator fraction yielded two bands on the gradient. The band of low density contained a mixture of endocrine, mucous and other cells. The third elutriator fraction yielded three bands on the gradient; the second band was removed and contained approx. 85–95% periodic acid–Schiff stain-positive cells and 5–15% periodic acid–Schiff stain-negative cells. The fifth elutriator fraction yielded three bands on the gradient, and the second and third bands were removed. The second band contained approx. 98% parietal cells and the third band contained virtually 100% pure chief cells. Cell characterization by light microscopy was performed as described previously (Seidler et al., 1989a,b). Mucous cells were identified by their large cytoplasmic granules that stain bright magenta red with the periodic acid–Schiff stain. The viability of the isolated cells was tested by Trypan Blue exclusion and by the observation of bright intracellular fluorescence with 2',7'-bis-(2-carboxy-



**Figure 4** Amplification of cDNA corresponding to the  $\gamma$ - and  $\delta$ -subunits of CaM kinase II in rat gastric cells

The cDNA was amplified with either a pair of primers yielding a 219 bp amplified sequence with  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -subtypes of CaM kinase II (lanes 1–7) or with primers specific for the  $\delta$ -subunit yielding a 309 bp sequence (lanes 8–10). The two outer lanes show markers the length of which is indicated by the numbers. After amplification of cDNA as described in the Experimental section, the unpurified material was cut with the following restriction enzymes (length of expected fragment for the subtypes indicated in parentheses): lane 1, none (219 bp); lane 2: *Bst*XI ( $\beta$  and  $\delta$  140/79); lane 3, *Eco*RV ( $\beta$  and  $\gamma$  132/87); lane 4: *Dra*II ( $\alpha$  156/63,  $\beta$  185/34); lane 5: *Hae*III ( $\alpha$  125/94,  $\beta$  120/67/32,  $\gamma$  125/94); lane 6, *Mae*II ( $\delta$  162/57); lane 7, *Xho*II ( $\gamma$  and  $\delta$  156/63); lane 8, none  $\delta$ -specific primers (309 bp); lane 9, *Dde*I ( $\delta$  154/100/55); lane 10, *Mae*II ( $\delta$  228/62/19).

ethyl)-5(6)-carboxyfluorescein (Herman et al., 1988) and was always greater than 95%.

#### Extraction of RNA and PCR

RNA was extracted from isolated cells as described by Chomczynski and Sacchi (1987). Total RNA (0.2–1  $\mu$ g) was reverse-transcribed using M-MuLV reverse transcriptase (Gibco) and buffers supplied with the enzyme, 100 pmol of random hexamer primers p(dN)<sub>6</sub> (Boehringer) in the presence of 40 units of placental RNase inhibitor (Promega). The RNA was heated to 90 °C for 3 min, placed immediately on ice, incubated at 18 °C for 15 min, then at 42 °C for 60 min and finally at 95 °C for 10 min.

The PCR primers for the CaM kinase II family were: left 5'-primer, 5'-CAGCAGGCTTGGTTTGGTTT-3'; right 3'-primer, 5'-TGTGTCCTTCTGGTGTATG-3'. The cDNA was heated

for 10 min at 95 °C. One unit of *Taq* polymerase (Perkin-Elmer-Cetus or Boehringer-Mannheim) was added with 3.8 mM MgCl<sub>2</sub> (final concentration) and 25 pmol of primers/100  $\mu$ l final volume. The cycle times were: 40 s 95 °C, 2.5 min 60 °C, 2 min 72 °C over 30 cycles. A 219 bp amplification product is predicted with a sequence derived from the catalytic regions of the  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits.

The CaM kinase II  $\delta$ -specific primer was: left, 5'-CCACCATTGAGGATGAAGAC-3'; right, 5'-CCATGTACTGTGTGAGCCGA-3'; both sequences are within the association domain. Cycling conditions were 3.8 mM MgCl<sub>2</sub>, 25 pmol of primers/100  $\mu$ l final volume, 40 s 95 °C, 2 min 55 °C, 2 min 72 °C, 30 cycles. A 309 bp amplification is expected with the  $\delta$ -subunit exclusively. For amplification of the  $\beta$ -subunit a specific right primer with the sequence 5'-GAGGCCGG-TGGACATTGT-3' was used which yields an amplification of 491 bp. The left primer was the family primer described above. This primer pair amplified the expected 491 bp cDNA fragment in rat brain and yielded 283, 140 and 68 bp fragments on restriction with *Bst*XI, a 404 and 87 bp fragment with *Eco*RV and 197, 185 and 109 bp fragments with *Dra*II as predicted from the nucleic acid sequence. Each amplification contained a control without prior reverse transcription and a sample that contained all components but neither RNA or DNA as negative controls.

After PCR, the samples were separated on a 1.5% (w/v) agarose gel. For restriction analysis the expected amplification products were isolated from agarose gels using the Mermaid kit (Bio 101) and were separated on polyacrylamide gels after digestion. Silver staining was used for detection. In order to confirm the sequences, the amplified DNA was blunt-end cloned into Bluescript and sequenced using the Sequenase II kit (USB). The sequences corresponded to the published sequences of the  $\gamma$ - and  $\delta$ -subunits of CaM kinase II.

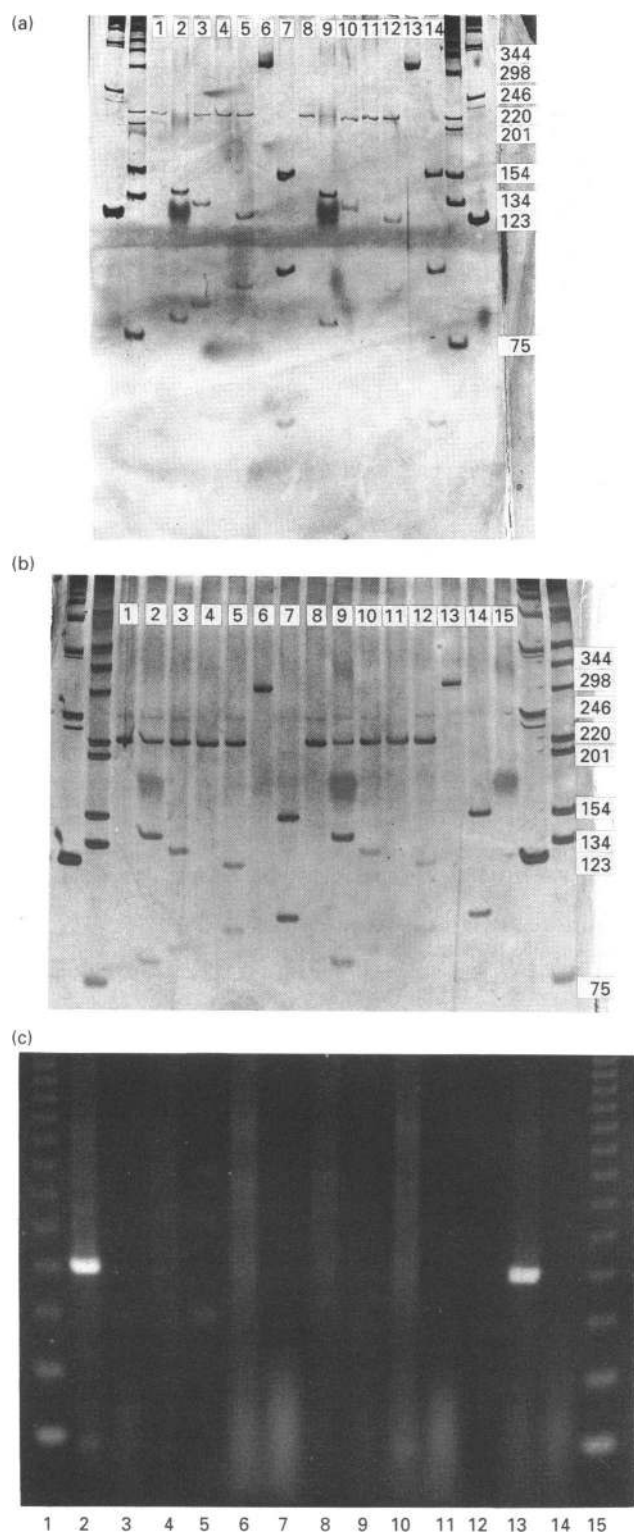
#### RESULTS

The present study was designed to identify the subunits of CaM kinase II present in rat gastric parietal cells by biochemical and molecular biological methods.

In order to determine whether rat gastric parietal cells possess a calmodulin-dependent protein kinase activity, we investigated the phosphorylation pattern of endogenous proteins observed in supernatants of parietal cells *in vitro*. A Ca<sup>2+</sup>- and calmodulin-dependent phosphorylation of a prominent 50 kDa and a 100 kDa protein was constantly observed in SDS/polyacrylamide gels. A weaker phosphorylation of a 60 kDa band was also detectable (results not shown).

The phosphorylated protein bands might represent either an autophosphorylated kinase or substrates of a calmodulin-dependent kinase. A 100 kDa phosphoprotein was previously described in parietal cell cytosol (Ottsdottir et al., 1987) and proposed to represent the CaM kinase III substrate (Nairn et al., 1985; Nygard et al., 1991). The 50 kDa band corresponds in size to the  $\alpha$ -subunit of CaM kinase II. The 60 kDa phosphorylated protein corresponds to the size of the  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits of CaM kinase II in rat brain. However, the presence of other phosphoproteins of identical size was also possible.

To assess calmodulin-binding proteins, <sup>125</sup>I-calmodulin overlays were performed in the presence or absence of Ca<sup>2+</sup> using a cytoplasmic preparation of rat parietal cells. As a reference, rat brain homogenate was used. In an experiment in the absence of Ca<sup>2+</sup>, performed as negative control, strong binding of <sup>125</sup>I-calmodulin was seen to proteins smaller than 30 kDa which was enhanced compared with in the presence of Ca<sup>2+</sup>. In the presence of Ca<sup>2+</sup>, <sup>125</sup>I-calmodulin labelled a 50 and 60 kDa band in rat



**Figure 5** Demonstration of mRNA for the  $\gamma$ - and  $\delta$ -subunit of CaM kinase II in purified gastric cell fractions

(a) Parietal (lanes 1–7) and chief (lanes 8–14) cells. (b) Mucous (lanes 1–7) and remaining, mixed (lanes 8–14) cells. Lanes 1–5 and 8–12 show amplifications of cDNA obtained with the CaM kinase II family primers; lanes 6, 7, 13 and 14,  $\delta$ -subunit-specific primers; lane 15, *Bst*XI without additions. The following restriction enzymes were used (numbers refer to lanes): 1 and 8, none; 2 and 9, *Bst*XI; 3 and 10, *Eco*RV; 4 and 11, *Dra*II; 5 and 12, *Hae*III; 6 and 13, none,  $\delta$  primers; 7 and 14, *Dde*I. The expected length of the fragments is indicated in the legend to Figure 3. (c) Demonstration of the absence of CaM kinase  $\beta$  in gastric cell subpopulations. The figure shows an agarose gel of fragments amplified with the  $\beta$ -specific primers yielding a

brain cytosol, and a 60 and 61 kDa doublet was observed in rat parietal cell supernatant (Figure 1).

This band also corresponded to the size of the 60 kDa phosphorylated cytosolic protein, raising the possibility that this represented an autophosphorylated calmodulin-dependent kinase. The  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits of the multifunctional CaM kinase II are thought to have sizes of 59–61 kDa (Bennet and Kennedy, 1987; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). The absence of a major calmodulin-binding protein at 50 kDa also supported the absence of the  $\alpha$ -subunit of CaM kinase II in gastric tissue as has been suggested by Fukunaga et al. (1990) on the basis of immunohistochemical data obtained with a polyclonal antibody to rat brain CaM kinase II.

Substrate-phosphorylation assays were performed to demonstrate the presence of CaM kinase II activity. A good substrate was autocamtide II, a peptide substrate highly specific for CaM kinase II which has been modelled after the auto-inhibitory domain of the  $\alpha$ -subunit (Hanson et al., 1989). However, this substrate also was phosphorylated in a Ca<sup>2+</sup>-dependent but calmodulin-independent manner in most preparations of enriched parietal cells. When the activity obtained in the presence of Ca<sup>2+</sup> and calmodulin was measured against that obtained with Ca<sup>2+</sup>, the  $K_m$  was 1  $\mu$ M, corresponding to that described for the rat brain enzyme (Figure 2a). To avoid interference by this activity, the Ca<sup>2+</sup>-dependent phosphorylation was subtracted from the Ca<sup>2+</sup>/calmodulin-dependent activity. A dose-response curve for calmodulin showed half-maximal stimulation at 70 nM calmodulin (Figure 2b). The calmodulin-dependent activity, but not the Ca<sup>2+</sup>-dependent but calmodulin-independent kinase activity, was inhibited by KN-62, a specific inhibitor of the CaM kinase II with an  $IC_{50}$  of 2  $\mu$ M, which yields a further argument for the presence of a calmodulin-dependent kinase of this family (Tokumitsu et al., 1990).

A monoclonal antibody against CaM kinase II  $\alpha$ -subunit (Hanson et al., 1989) did react with the kinase in rat brain cytosol but not with cytosolic proteins from enriched parietal cells. This agrees with the absence of a 50 kDa band in the calmodulin overlay typical of the  $\alpha$ -subunit (results not shown).

In order to identify the subunit of CaM kinase II present in the rat mucosal cells, RNA was extracted and reverse-transcribed before amplification by the PCR technique. The pair of primers was chosen in such a way that  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -subtypes of the CaM-kinase II were expected to be amplified, yielding a band of 219 bp in silver-stained polyacrylamide gels or in ethidium bromide-stained agarose gels. No amplification was observed with DNA preparations, indicating the presence of introns in genomic DNA. The subtypes were then identified by analysis of the restriction-enzyme pattern for each type of kinase. The ability to amplify all subtypes was tested in rat brain cDNA and yielded the expected restriction patterns for  $\alpha$ ,  $\gamma$  and  $\delta$  (Figure 3a) with six different restriction enzymes. In particular, *Dra*II was specific for the  $\alpha$  sequence and did not cut  $\gamma$  or  $\delta$  sequences of CaM kinase II. *Dra*II yielded the expected 156 and 63 bp fragments for the  $\alpha$ -subunit while bands of 185 and 34 bp expected for  $\beta$  were not easily discernible (Figure 3a). Using *Hae*III, a  $\beta$ -specific band was present at 67 bp in rat brain

491 bp sequence as described in Figure 3(b). Lane 1, 123 ladder; lane 2, cDNA from rat brain; lane 3, RNA from rat brain without reverse transcription; lane 4, cDNA from the mixed gastric cell fraction; lane 5, RNA from the mixed gastric cell fraction without reverse transcription; lane 6, cDNA from mucous cells; lane 7, RNA from mucous cells without reverse transcription; lane 8, cDNA from chief cells; lane 9, RNA from chief cells without reverse transcription; lane 10, cDNA from parietal cells; lane 11, RNA from parietal cells without reverse transcription; lane 12, blank with reverse transcription but without RNA or DNA; lane 13, cDNA from rat brain; lane 14, RNA from rat brain without reverse transcription; lane 15, 123 ladder.

(Figure 3a) showing that some amplification of the  $\beta$  sequence was achieved. In order to avoid ambiguities with regard to the  $\beta$ -subtype, a  $\beta$ -specific right primer was employed together with the left 'family-primer', which yielded the expected 491 bp fragment in rat brain. On restriction with *Bst*XI, *Eco*RV and *Dra*II, the predicted fragments resulted (Figure 3b).

In rat gastric cell extracts, no cleavage was obtained with *Dra*II (Figure 4, lane 4), suggesting the absence of  $\alpha$ -subunit mRNA. A restriction enzyme specific for the  $\gamma$ - and  $\delta$ -subtypes, i.e. *Xho*II (Figure 4, lane 7), yielded the expected bands and completely digested the 219 bp sequence whereas enzymes digesting only  $\gamma$  or  $\delta$  sequences (*Bst*XI, *Eco*RV, *Hae*III and *Mae*II) yielded incomplete digests of the 219 bp sequence, suggesting that both subtypes were present in gastric cells. A primer pair specific for the  $\delta$  subunit which amplified a sequence located in the association domain of the kinase (Schulman and Lou, 1989) also yielded the predicted band of 309 bp and the expected restriction fragments with *Dde*I and *Mae*II (Figure 4).

As the PCR technique is highly sensitive, the possibility existed that the enzyme was present in a small subpopulation of gastric cells only. Therefore gastric mucosal cells were purified according to size using counterflow elutriation as a first step followed by separation according to cell density in a continuous gradient of Percoll. This allowed almost complete purification of parietal, chief and mucous cells. A fourth mixed fraction of small unidentified cells was also obtained. RNA extracted and reverse-transcribed from these cells was analysed as described above. The restriction pattern showed the presence of the  $\gamma$ - and  $\delta$ -subtypes of the CaM kinase II in all cell preparations, and no restriction patterns corresponding to the  $\alpha$ - and  $\beta$ -subunit were apparent (Figures 5a and 5b). Moreover, the  $\beta$ -specific primer pair also did not amplify cDNA from purified gastric cell populations whereas rat brain cDNA was readily amplified (Figure 5c).

Several studies have investigated the types of CaM kinase II occurring in peripheral tissues. Tobimatsu and Fujisawa (1989) demonstrated on the basis of mRNA distribution that peripheral tissues primarily contain  $\gamma$  and  $\delta$  subtypes. These enzymes have molecular masses of 59–61 kDa. A 51 and 52 kDa enzyme was purified from rat and rabbit liver and lung (Nishimura et al., 1988). Heterogeneity of peripheral subtypes was also suggested by earlier studies (Shenolikar et al., 1986). The PCR technique combined with analysis of restriction patterns, as presented here, is a relatively simple and reliable method of determining the distribution of CaM kinase II subtypes without the danger of missing subtypes, as may occur when separate pairs of primers that may differ in efficiency of amplification are used.

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