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# Protein kinase C-α attenuates cholinergically stimulated gastric acid secretion of rabbit parietal cells

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**1** The phorbolester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C (PKC), inhibits cholinergic stimulation of gastric acid secretion. We observed that this effect strongly correlated with the inhibition of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) activity in rabbit parietal cells.

**2** The aim of this study was to specify the function of PKC-α in cholinergically stimulated $H^+$ secretion. PKC-α represents the only calcium-dependent PKC isoenzyme that has been detected in rabbit parietal cells.

**3** Gö 6976, an inhibitor of calcium-dependent PKC, concentration-dependently antagonized the inhibitory effect of TPA, and, therefore, revealed the action of PKC-α on carbachol-induced acid secretion in rabbit parietal cells.

**4** TPA exerted no additive inhibition of carbachol-stimulated acid secretion if acid secretion was partially inhibited by the potent CaMKII inhibitor 1-[*N*,*O*-bis(5-isoquinolinsulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine (KN-62).

**5** Since both kinase modulators, TPA and KN-62, affected no divergent signal transduction pathways in the parietal cell, an *in vitro* model has been used to study if PKC directly targets CaMKII. CaMKII purified from parietal cell-containing gastric mucosa of pig, was transphosphorylated by purified cPKC containing PKC-α up to 1.8 mol $P_i$ per mol CaMKII *in vitro*. The autonomy site of CaMKII was not transphosphorylated by PKC.

**6** The phosphotransferase activity of the purified CaMKII was *in vitro* inhibited after transphosphorylation by PKC if calmodulin was absent during transphosphorylation. Attenuation of CaMKII activity by PKC showed strong similarity to the downregulation of CaMKII by basal autophosphorylation.

**7** Our results suggest that PKC-α and CaMKII are closely functionally linked in a cholinergically induced signalling pathway in rabbit parietal cells. We assume that in cholinergically stimulated parietal cells PKC-α transinhibits CaMKII activity, resulting in an attenuation of acid secretion.
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**Keywords:** Calcium; $Ca^{2+}$/calmodulin-dependent protein kinase; carbachol; gastric acid secretion; indolocarbazole; parietal cell; phorbol ester; protein kinase C; protein kinase inhibition

**Abbreviations:** AP, dimethylamine-aminopyrine; BSA, bovine serum albumin; CaMKII, $Ca^{2+}$/calmodulin-dependent protein kinase II; carbachol, (2-hydroxyethyl)trimethylammonium chloride carbamate; cPKC, conventional protein kinase C; EGF, epidermal growth factor; ECL, enhanced chemiluminescence; GF 109203X, 2-[l-(3-dimethylaminopropyl)-lH-indol-3-yl]-3-(lH-indol-3-yl)-maleimide; Gö 6976,12-(2-cyanoethyl)-6, 7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine; PKC, protein kinase C or $Ca^{2+}$-sensitive phospholipid-dependent protein kinase; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

## Introduction

Besides histamine and gastrin, acetylcholine is an important secretagogue to induce $H^+$ secretion (reviewed in Urushidani & Forte, 1997; Schubert, 2002). Activation of the muscarinic $M_3$ receptor stimulates phospholipase C to cleave phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacylglycerol (Chew & Brown, 1986; Pfeiffer *et al.*, 1990; Seidler & Pfeiffer, 1991; Kajimura *et al.*, 1992). The generated inositol trisphosphate mobilizes $Ca^{2+}$ from the endoplasmatic reticulum. In parietal cells, free intracellular $Ca^{2+}$ stimulates signalling pathways involving protein kinase C (PKC) (Brown & Chew, 1987; Chiba *et al.*, 1989; Nandi *et al.*, 1996; reviewed in Fährmann, 2000) and $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) (Tsunoda *et al.*, 1992; Fährmann *et al.*, 2002a,b), respectively, which are

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both abundant in gastric parietal cells (Mayer *et al.*, 1994; Nandi *et al.*, 1996, 1999; Chew *et al.*, 1997; Fährmann *et al.*, 1998; 1999; 2002a; Fährmann & Pfeiffer, 2000).

Both PKC and CaMKII are multifunctional kinases, which are present in many cell types (reviewed in Nishizuka, 1992; Braun & Schulman, 1995; Kanashiro & Khalil, 1998). cPKC and CaMKII, respectively, are *in vivo* stepwise activated in a spatio-temporal manner (Hanson & Schulman, 1992; Meyer *et al.*, 1992; Hanson *et al.*, 1994; Mukherji & Soderling, 1994; Newton, 1997; Oancea & Meyer, 1998; Brocke *et al.*, 1999). CaMKII appears to have an obligatory role in acid secretion because inhibition of CaMKII activity blocks gastric acid secretion (Tsunoda *et al.*, 1992; Fährmann *et al.*, 2002a). We recently showed that CaMKII is activated and translocated to the secretory apical membrane during carbachol-induced acid release (Fährmann *et al.*, 2002a).

In contrast to the stimulatory effect of the CaMKII, PKC seems to inhibit the major acid-secreting pathway rather than having a promoting role in secretagogue-induced acid formation. Although either stimulating or inhibitory effects of PKC were reported, most studies showed its inhibitory role on acid secretion (Anderson & Hanson, 1985; Muallem *et al.*, 1986; Beil *et al.*, 1987; Brown & Chew, 1987; Chiba *et al.*, 1989; Nandi *et al.*, 1996; Wang *et al.*, 1996; Kopp & Pfeiffer; 2000; Beales & Calam, 2001; Fährmann *et al.*, 2002b; reviewed in Fährmann, 2000). Intracellular inhibition or activation of PKC was mostly analyzed pharmacologically utilizing cell-permeable inhibitors or activators such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Anderson & Hanson, 1985; Muallem *et al.*, 1986; Beil *et al.*, 1987; Brown & Chew, 1987; Chiba *et al.*, 1989; Nandi *et al.*, 1996; Kopp & Pfeiffer, 2000, Fährmann *et al.*, 2002b). Since the specific roles of PKC isoforms in parietal cells are not well understood, the here results presented address the function of PKC-α, the only member of cPKC (conventional PKC) detected in rabbit parietal cells by immunoblotting (Nandi *et al.*, 1996; 1999; Chew *et al.*, 1997), in cholinergically stimulated $H^+$ release. PKC isoforms are sequentially activated by calcium and diacylglycerol signals (Newton, 1997; Oancea & Meyer, 1998). PKC-α has been observed as an activator of secretion, for example, $Ca^{2+}$-induced secretion of α- and dense-core granules in platelets (Yoshioka *et al.*, 2001), or secretion of amyloid precursor protein (Benussi *et al.*, 1998). However, in the case of rabbit gastric parietal cells, our data indicate an inhibitory function of PKC-α during cholinergically induced acid secretion.

## Methods

### *Chemicals and drugs*

Calmodulin, 2-[l-(3-dimethylaminopropyl)-lH-indol-3-yl]-3-(lH-indol-3-yl)-maleimide (GF 109203X, bisindolyl-maleimide I, Gö 6850), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö 6976), TPA and 1-[*N*, *O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine (KN-62) were obtained from Calbiochem (Bad Soden, Germany). ATP containing 370 GBq $mol^{-1}$ γ-[$^{32}$P]ATP was from ICN (Meckenheim, Germany). [$^{14}$C]AP ([dimethylamine-$^{14}$C]aminopyrine) of 3.92 GBq $mol^{-1}$ was obtained from Amersham Biosciences (Freiburg, Germany). CaMKII substrate autocamtide-II of sequence KKALRRQETVDAL, leupeptin, and pepstatin A were purchased from Bachem (Heidelberg, Germany). PKC substrate peptide of sequence VRKRTLRRL (American Peptide, St Louis, U.S.A.) was derived from the EGF receptor according to Hunter *et al.* (1984). Pefabloc SC was from Roche (Strasburg, France). All other fine chemicals were from Sigma (Deisenhofen, Germany) if not mentioned otherwise.

### *Primary culture of rabbit gastric parietal cells*

Isolation of rabbit gastric parietal cells was performed as described (Fährmann *et al.*, 2002b). In brief, after high-pressure perfusion of the stomach *in situ*, the gastric mucosa was scraped off, and disintegrated by both pronase and collagenase. Gastric parietal cells were enriched by elutriation utilizing a Beckman JM6-C centrifuge with a JE-5.0 quick assembly rotor (Beckman, München, Germany), and subsequent separation by centrifugation in a Nycodenz (Nycomed, Oslo, Norway) step gradient. Highly enriched parietal cells of 95 – 98% purity were transferred to DMEM-Ham's F-12 medium containing 2 mg $ml^{-1}$ bovine serum albumin, (BSA) 800 nM insulin, 5 μg $ml^{-1}$ transferrin, 5 ng $ml^{-1}$ sodium selenite, 10 nM hydrocortisone, 8 nM epidermal growth factor EGF, 5 μg $ml^{-1}$ geneticin, 50 μg $ml^{-1}$ novobiocin, 100 μg $ml^{-1}$ gentamicin, and 10 μg $ml^{-1}$ phenol red. Parietal cells were cultured in 24-well plates precoated with Matrigel (Becton Dickinson, Bedford, MA, U.S.A.).

### *Assessment of acid formation with [$^{14}$C]aminopyrine uptake*

The formation of $H^+$ of cultured rabbit parietal cells was indirectly determined by uptaking [$^{14}$C]AP as previously described (Fährmann *et al.*, 2002b). Parietal cells were incubated in oxygen-saturated buffer A ((mM) HEPES 10, pH 7.4, plus NaCl 114.4, KC1 5.4, $Na_2HPO_4$ 5, $NaH_2PO_4$ 1, $MgSO_4$ 1.2, $CaCl_2$ 2, 2 mg $ml^{-1}$ BSA, glucose 10, dithiothreitol 0.5, pyruvate 1) in the absence or presence of diverse protein kinase activators or inhibitors with subsequent stimulation by various concentrations of ((2-hydroxyethyl) trimethylammonium chloride carbamate) (carbachol) (0 – 0.1 mM) for 45 min. Incorporation of $^{14}$C was measured in a β-scintillation counter after a two-times wash and lysis of the cells. Basal acid secretion was determined by inhibition with 0.1 mM ranitidine. The nonspecific [$^{14}$C]AP accumulation was obtained by blocking acid secretion with omeprazol in control assays (10 μM; AstraZeneca, Wedel, Germany). The nonspecific background was subtracted from each specific value.

### *Preparation of membrane fraction of parietal cells*

Parietal cells ($5 \times 10^6$ cells $ml^{-1}$) were lysed by short-pulse sonication in buffer B ((mM) HEPES/NaOH 40, pH 7.4, with EGTA 10, EDTA 2, dithiothreitol 1, leupeptin 0.1, pepstatin A 1 μg $ml^{-1}$, pefabloc SC 0.1, aprotinin 0.25). The lysate was separated in the cytosolic and the microsomal fraction by centrifugation at $100{,}000 \times g$ for 1 h at 4°C. The microsomal fraction was resuspended in buffer B containing 0.5% (v $v^{-1}$) Triton X-100. After incubation on ice for 30 min, the suspension was repelleted at $100{,}000 \times g$ for 1 h at 4°C. The supernatant was designated as 'membrane fraction'. The

protein concentration was determined according to Stoschek (1990).

### *Purification of protein kinases*

PKC-α was contained in purified cPKC of rat forebrain according to Go *et al.* (1987), and was purchased from Roche. The cPKC preparation was certified by the manufacturer that proteases within the PKC preparation were not detectable. We detected PKC-α but neither possibly copurified CaMKII, PKC-ε, nor calmodulin with appropriate antibodies in the cPKC preparation (data not shown). We purified a hetero-oligomeric CaMKII from the homogenate of the corpus of gastric mucosa of freshly slaughtered pigs (*Sus scrofa f. domestica* L.) to apparent homogeneity according to Fährmann and Pfeiffer (2000). Gastric mucosa is represented by 50 – 60% of the mass of parietal cells (Hogben *et al.*, 1974). Therefore, most of the isolated CaMKII has been specific to parietal cells. CaMKII activity was measured as [$^{32}$P]phosphoryl incorporation into the CaMKII-specific substrate peptide autocamtide-II. For calculating molar ratios, an average molecular mass of 60 kDa was used for CaMKII subunits of porcine gastric mucosa according to Fährmann & Pfeiffer (2000), and 77 kDa for cPKC of rat brain (Kanashiro & Khalil, 1998).

### *Protein kinase assays*

Assays were performed in a 25 μl reaction volume for gel analysis, or in a 50 μl volume to determine kinase activity on phosphocellulose paper. We used a PKC-specific substrate peptide (VRKRTLRRL) (Hunter *et al.*, 1984) to determine PKC phosphotransferase activity in buffer C ((mM) HEPES/NaOH 25, pH 7.4, plus phosphatidylserine 20 μg ml$^{-1}$, diacylglycerol 25 μg ml$^{-1}$, $MgCl_2$ 10, $CaCl_2$ 0.001, EGTA 0.0005, $ZnCl_2$ 0.01, dithiothreitol 0.5, 0.1 μg μl$^{-1}$ BSA), with γ-[$^{32}$P]ATP (5 Bq pmol$^{-1}$) at 0.1 mM ATP, and optional 60 μMPKC substrate peptide.

The two-step assay presented in Figure 4 was performed in buffer C without PKC substrate peptide and BSA. In the first step, aliquots of purified gastric mucosal CaMKII (10 ng) were phosphorylated in the presence of different PKC/CaMKII molar ratios (0 – 3.0 mol mol$^{-1}$) for 10 min in a reaction volume of 25 μl. In our experiments, we analyzed the concentration-dependent (Figures 3a, b and 4) rather than the time-dependent phosphorylation of CaMKII by PKC since CaMKII itself exhibits a time-dependent basal autophosphorylation (Figure 5). Therefore, a time-dependent phosphorylation of CaMKII by PKC would be nearly impossible to analyze. The second step was a test of CaMKII phosphotransferase activity, performed as a standard assay with $Ca^{2+}$/calmodulin (0.1 mM/40 nM), autocamtide-II (60 μM) as substrate, and bisindolylmaleimide GF 109203X (0.1 μM) to inhibit PKC activity specifically (Toullec *et al.*, 1991). The total reaction volume at this state was 50 μM. In the presence of 0.1 μM GF 109203X, PKC activity was specifically inhibited down to 4% activity of the uninhibited state (100%) whereas gastric mucosal CaMKII exhibited still 96% phosphotransferase activity compared to its activity in the absence of GF 109203X (100%) (data not shown).

The activity of CaMKII was measured by transphosphorylation of autocamtide-II (60 μM) either in the presence of buffer C for the two-step assay, or in buffer D ((mM) HEPES/NaOH 25, pH 7.4, with $CaCl_2$ 0.2, EGTA 0.1, $MgCl_2$ 10, dithiothreitol 0.5, BSA 0.1 μg μl$^{-1}$), each buffer with γ-[$^{32}$P]ATP (5 Bq pmol$^{-1}$) at 100 μM ATP and optional 40 nM calmodulin. Autophosphorylation of CaMKII was carried out in buffer D in the presence at 100 μM ATP with γ-[$^{32}$P]ATP (5 Bq pmol$^{-1}$) and optional 40 nM calmodulin.

For gel analysis, the reactions were terminated with an SDS-β-mercaptoethanol disruption buffer, and subsequently analyzed by SDS – polyacrylamide gel electrophoresis. Gels were dried and exposed to Hyperfilm MP (Amersham Biosciences) to visualize [$^{32}$P]-labeled bands, or to cut out radioactively labeled bands for counting in a β-scintillation counter. Autoradiographies were quantified by densitometry utilizing the 'Personal Densitometer SI' and its program 'ImageQuaNT' (Molecular Dynamics Inc., Sunnyvale, U.S.A.).

### *Immunoblots*

All immunoblotting steps followed the protocol according to Fährmann *et al.* (2002b). Membrane proteins (15 μg) of rabbit parietal cells were separated in 12.5% acrylamide/2.67% bisacrylamide (mass volume$^{-1}$) SDS-gels, followed by a transfer onto a polyvinylidene difluoride membrane (Pierce, Rockford, IL, U.S.A.). A rabbit polyclonal antiactive (rat)CaMKII antibody against phosphorylated threonine of the autonomy site (code V1111; Promega, Mannheim, Germany) was utilized to detect activated CaMKII (1 : 5000). Each antibody, including secondary antibody, was incubated for 1 h. After washing, antibody binding was visualized by the enhanced chemiluminescence (ECL) detection plus system (Amersham Biosciences). The immunolabeled CaMKII bands on ECL films were quantified as described above for autoradiographies.

### *Statistical analysis and calculation*

Data values are shown as means ± s.e.m. of at least three independent experiments ($n = 3$); each assay of an experiment was performed at least three times. The statistical significance of differences was determined by analysis of variance (ANOVA) with the *post hoc* Dunnett test. Differences were regarded as significant at $P < 0.05$. Values of $IC_{50}$ were calculated by using 'Grafit' (Erithacus Software Ltd., U.K.).

## Results

### *TPA inhibits both carbachol-induced acid secretion and CaMKII activity in rabbit parietal cells*

Both activation of PKC and inhibition of CaMKII, respectively, lead to a decrease in cholinergically stimulated acid secretion. A very strong correlation between inhibition of CaMKII and $H^+$ secretion has been observed. Comparison of inhibition of kinase activity of CaMKII purified from parietal cell-containing mucosa to inhibition of carbachol-induced [$^{14}$C]AP accumulation by KN-62 (Hidaka & Yokokura, 1996) each, showed a very similar $IC_{50}$ of KN-62 (39 to 32 nM) as well as a nearly identical sigmoidal curve of inhibition (Figures 1a, b). There has also been a strong correlation between both

TPA-dependent inhibitions of carbachol-stimulated acid secretion and of CaMKII activity in parietal cells (Figure 1c). To show if TPA directly affects the phosphotransferase activity of CaMKII, we incubated purified CaMKII with various concentrations of TPA (0 – 1 μM). We did not observe any significant effect of TPA on CaMKII activity (data not shown).

An involvement of PKC-α was shown by the indolocarbazole Gö 6976 (0 – 1 μM) (Figure 2a). Gö 6976 specifically inhibits calcium-dependent PKC isoforms (Martiny-Baron *et al.*, 1993). PKC-α appears to be the only member of cPKCs relevant to rabbit parietal cells. Therefore, effects of Gö 6976 on parietal cell function should be explained by specific inhibition of PKC-α. Gö 6976 (0 – 1 μM) increased carbachol-stimulated [$^{14}$C]AP uptake with a maximum increase to 138.5 ± 11.3%, at 10 μM (Figure 2a). The inhibition of the carbachol-evoked [$^{14}$C]AP uptake by TPA (1 μM) was concentration-dependently reversed with Gö 6976 (0 – 1 μM) (Figure 2a). Full compensation of the TPA effect was achieved at 1 μM Gö 6976. Remarkably, carbachol-induced acid secretion was 1.8-fold more stimulated if Gö 6976 (10 μM) and TPA (1 μM) were combined. The compensatory effect of Gö 6976 on TPA-inhibited $H^+$ secretion was antagonized by the CaMKII inhibitor KN-62 (20 μM) (Figure 2a).

To support our assumption of a close functional linking of PKC-α and CaMKII to the same signaling pathway for acid secretion, an experiment has been carried out to combine the effects of TPA and KN-62 on acid secretion. In the case of divergent signal transduction pathways, activation of PKC and inhibition of CaMKII should be additive to inhibit $H^+$ secretion. Carbachol-stimulated acid secretion was partially inhibited by preincubation with KN-62. Subsequent concentration-dependent treatment with TPA exhibited no additional inhibition on acid secretion (Figure 2b). This suggests that TPA and KN-62 may affect the same target, presumably CaMKII, in carbachol-evoked acid secretion signaling. At high concentrations of TPA (10 nM – 1 μM), inhibition by KN-62 was not additive to the TPA effect.

### *Phosphorylation of CaMKII is increased in the presence of PKC*

To corroborate the idea that PKC-α suppresses CaMKII activity, we used purified cPKC containing PKC-α of rat forebrain to phosphorylate purified CaMKII of parietal cell-containing hog gastric mucosa with different concentrations of PKC expressed as a molar ratio of PKC/CaMKII (0 – 3.0 mol mol$^{-1}$) at a constant concentration of CaMKII. The phosphorylation of CaMKII up to 1.8 mol $P_i$ per mol CaMKII

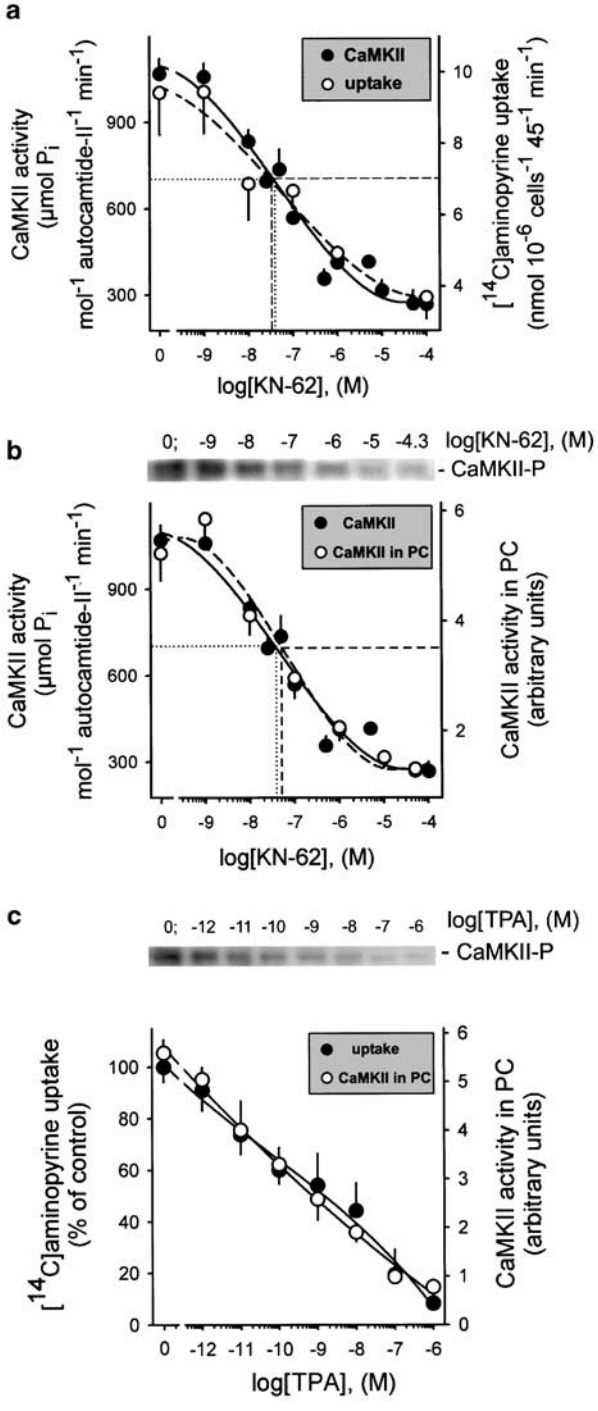


**Figure 1** Comparison of the inhibition of gastric acid secretion to the inhibition of gastric CaMKII activity by KN-62. (a) The potent and cell-permeable inhibitor of CaMKII, KN-62 (0 – 10$^{-4}$ M), was used to inhibit carbachol (0.1 μM)-stimulated acid secretion of cultured rabbit parietal cells (PC) (open points) as well as the phosphotransferase activity of purified CaMKII of PC-containing, hog gastric mucosa *in vitro* (black points). The $IC_{50}$ (39 nM; dotted line) of KN-62 to inhibit isolated CaMKII was very similar to the $IC_{50}$ (32 nM; broken line) to inhibit acid secretion. (b) Inhibition of purified CaMKII activity (black points) by KN-62 was very similar to the inhibition of intracellular CaMKII activity (open points) in carbachol (0.1 μM)-stimulated PC. Dotted line as in (a), broken line shows the $IC_{50}$ of 41 nM KN-62 for inhibition of CaMKII in PC. The immunoblot shows activated CaMKII (CaMKII-P) of the PC membrane after concentration-dependent treatment of PC with KN-62, and subsequent stimulation by carbachol (0.1 μM). (c) Cultured PC were incubated with various concentrations of TPA (0 – 1 μM) before stimulation by carbachol (0.1 μM). [$^{14}$C]AP uptake of PC (black points) or intracellular activity of CaMKII (open points) was assessed. Results were commonly plotted to compare the effects of TPA. Activated CaMKII (CaMKII-P) was detected in parietal cell membrane by immunoblot analysis. [$^{14}$C]AP uptake of 100% corresponds to 9.4 nmol 10$^{-6}$ cells$^{-1}$ 45$^{-1}$ min$^{-1}$. Vertical bars show s.e.m. ($n$ = 4).

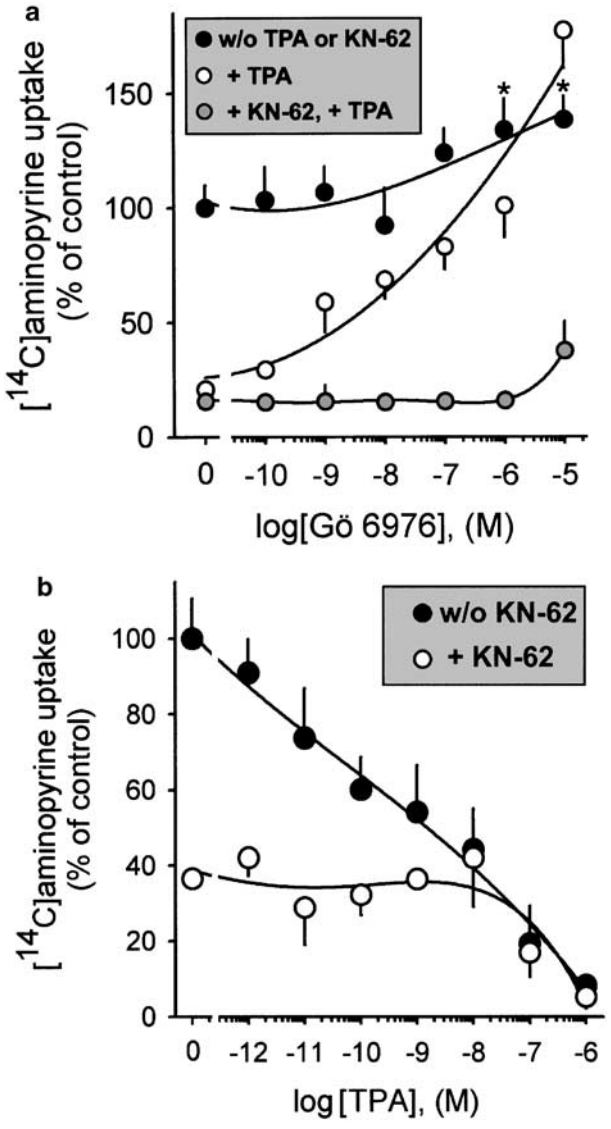


**Figure 2** TPA, an activator of PKC, dose-dependently inhibits carbachol-induced acid secretion but has no effect if CaMKII was inhibited by KN-62. (a) Cultured PC were blocked for carbachol (0.1 mM)-stimulated $H^+$ secretion by TPA (1 $\mu$M) (open and gray-filled points). The inhibition was reversed by Gö 6976 (0 – 10 $\mu$M) in a dose-dependent manner (open points). The compensatory effect of Gö 6976 on TPA-treated cells was inhibited by KN-62 (20 $\mu$M) (gray-filled points). As control, the effect of Gö 6976 on the carbachol-induced [$^{14}$C]AP accumulation was assessed (black points). [$^{14}$C]AP uptake of 100% corresponds to 16.2 nmol $10^{-6}$ cells$^{-1}$45$^{-1}$ min$^{-1}$. *Significantly different from carbachol alone, $P<0.05$. (b) Effect of TPA on partially KN-62-inhibited [$^{14}$C]AP accumulation was determined. Cultured PC were preincubated with various concentrations of either TPA (0 – 1$\mu$M) (black points), or both KN-62 (20 $\mu$M) and TPA (0 – 1 $\mu$M) (open points) for 30 min before carbachol induction (0.1 $\mu$M) for 45 min. [$^{14}$C]AP uptake of 100% corresponds to 9.4 nmol $10^{-6}$ cells$^{-1}$ 45$^{-1}$min$^{-1}$.

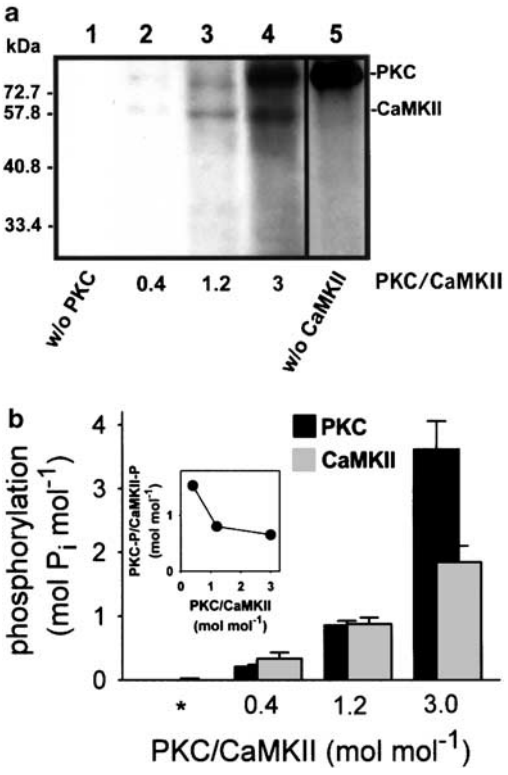


**Figure 3** Phosphorylation of purified hetero-oligomeric CaMKII in the presence of purified PKC. (a) Purified CaMKII aliquots (each lane 10 ng, except lane 5 without CaMKII) from PC-containing hog gastric mucosa, and purified cPKC containing PKC-$\alpha$ of rat forebrain were incubated under PKC phosphorylation conditions in the absence of calmodulin, with different PKC/CaMKII molar ratios per assay for 10 min in a reaction volume of 25 $\mu$l: lane 1, no PKC; lane 2, 0.4 mol mol$^{-1}$; lane 3, 1.2 mol mol$^{-1}$; lane 4, 3.0 mol mol$^{-1}$; lane 5, no CaMKII. Purified CaMKII exhibited no phosphorylation in the presence of PKC phosphorylation conditions (lane 1) but in the presence of PKC (lanes 2 – 4). Additionally, PKC showed autophosphorylation activity (lanes 2 – 5). Autophosphorylated PKC showed no phosphorylation in the range of CaMKII molecular mass (lane 5). The performance range of each protein kinase is indicated. (b) Bands of each lane as performed in (a) of PKC and CaMKII, respectively, were excised from the corresponding gels and counted for radioactivity. The relatively most efficient molar ratio of PKC/CaMKII for CaMKII phosphorylation by PKC-$\alpha$ was at 0.4 mol mol$^{-1}$ (inset). At 1.2 mol mol$^{-1}$, about 1 mol phosphoryl per mol CaMKII was incorporated. * Absence of PKC; PKC-P, phosphorylated PKC; CaMKII-P, phosphorylated CaMKII.

was dependent on the concentration of PKC (Figure 3a, lanes 2 – 4). CaMKII phosphorylation was most efficient at 0.4 mol PKC mol$^{-1}$ CaMKII$^{-1}$ (Figures 3a, b, inset). At nearly equimolar concentrations of PKC and CaMKII (1.2 mol mol$^{-1}$), 0.9 $\pm$ 0.1 mol phosphoryl per mol CaMKII was incorporated (Figure 3b). In the absence of PKC, no CaMKII phosphorylation was detectable (Figure 3a, lane 1). A control assay of PKC autophosphorylation showed no phosphorylations in the range of CaMKII isoform sizes (Figure 3a, lane 5). The results demonstrate that at a given time point, the degree of phosphorylation of CaMKII is strongly related to the amount of PKC present in the assay.

### *PKC-dependent modulation of CaMKII activity*

In a subset of experiments, the response of the phosphotransferase activity of CaMKII on transphosphorylation by PKC was assessed. Utilizing the purified enzymes, CaMKII was incubated with PKC, either in the presence or absence of

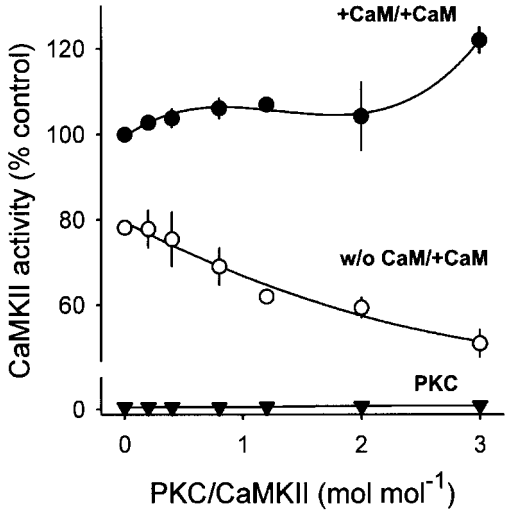


**Figure 4** Modulation of the CaMKII activity by PKC. As described in the 'Methods' section, a two-step assay was performed to determine the effect of cPKC containing PKC-α on the preparation of purified CaMKII. PKC transphosphorylation of CaMKII was performed in the presence (black points) or absence (open points) of calmodulin. The calmodulin-stimulated CaMKII transphosphorylation activity was inhibited to 78.2% activity after autophosphorylation in the absence of calmodulin for 10 min (first value of curve of open points) (see also Figure 5c). The background of PKC transphosphorylation of autocamtide-II was ⩽2% (black triangles), and subtracted from specific values. +CaM/+CaM, $Ca^{2+}$/calmodulin was present during the first and the second step; w/o CaM/+CaM, $Ca^{2+}$/calmodulin was only present during the second step of the assay.

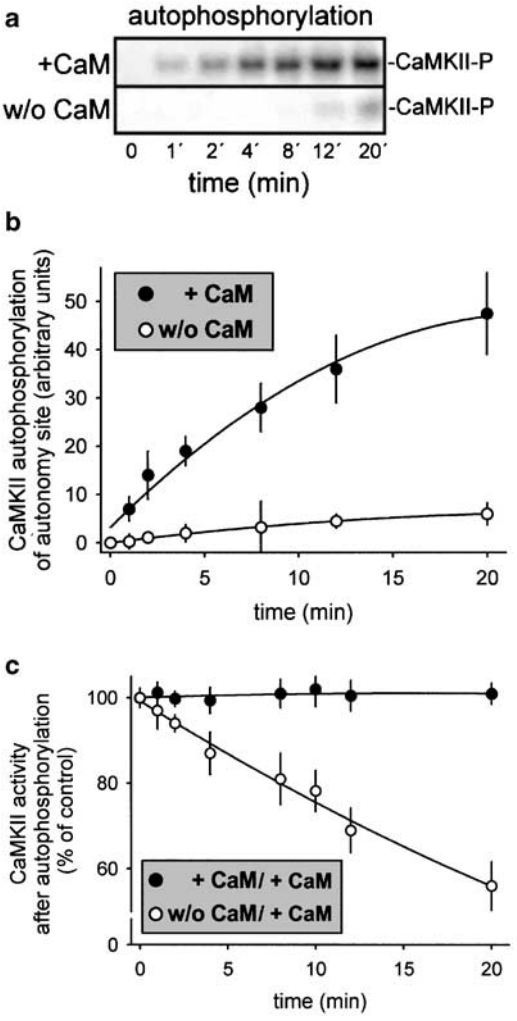


**Figure 5** Basal autophosphorylation time-dependently inactivates CaMKII transphosphorylation activity. (a) CaMKII purified from pig gastric mucosa was time-dependently (0 – 20 min) autophosphorylated either in the presence (+CaM) or absence (w/o CaM) of $Ca^{2+}$/calmodulin, each at pH 7.4 and 0.1 mM ATP. CaMKII, which autophosphorylated at threonine of the autonomy site (CaMKII-P), was detected with a polyclonal antiactive CaMKII antibody. Immunolabeling was visualized by the ECL system. (b) The kinetics (0 – 20 min) of autophosphorylation at the autonomy site of purified CaMKII either in the presence (black points) or absence (open points) of calmodulin is presented. ECL films were densitometrically quantified. (c) Autophosphorylation of purified gastric mucosal CaMKII in the absence (black points) or presence (open points) of calmodulin was followed by a test of CaMKII phosphotransferase activity towards autocamtide-II (60 μM). Vertical bars represent s.e.m. The inset explains the two-step assays: +CaM/+CaM, $Ca^{2+}$/calmodulin was present during autophosphorylation and subsequent transphosphorylation of substrate; w/o CaM/+CaM, absence of $Ca^{2+}$/calmodulin during autophosphorylation but presence during transphosphorylation.

$Ca^{2+}$/calmodulin. After incubation, we monitored the CaMKII phosphotransferase activity in the presence of $Ca^{2+}$/calmodulin, while PKC activity was simultaneously inhibited with GF 109203X (Toullec *et al.*, 1991) (Figure 4). Since the time-dependent autophosphorylation of CaMKII would interfere, it was not possible to perform a true time-dependent PKC phosphorylation of CaMKII. Instead, experiments were carried out at various concentrations of PKC and a constant time of 10 min. Basal autophosphorylation of CaMKII, that is, autophosphorylation in the absence of both calmodulin and PKC, resulted in a partial inactivation of the CaMKII phosphotransferase activity. We found only 78.2% of $Ca^{2+}$/calmodulin-stimulated CaMKII activity after 10 min of basal autophosphorylation compared to 100% control activity after 10 min autophosphorylation in the presence of calmodulin (Figure 4, value at 0 mol $mol^{-1}$ without calmodulin preincubation; Figure 5c).

After PKC phosphorylation of CaMKII in the presence of calmodulin, the CaMKII phosphotransferase activity was relatively unaffected over a wide range with a moderate increase to 122.1±7.2% CaMKII activity at the highest PKC/CaMKII ratio of 3.0 mol $mol^{-1}$ compared to the activity at 0 mol $mol^{-1}$ (100%) (Figure 4). In the absence of calmodulin, however, the CaMKII activity was dose-dependently inhibited by PKC transphosphorylation between 0 and 3.0 mol PKC $mol^{-1}$ $CaMKII^{-1}$. At equimolar concentrations of PKC and CaMKII (0.8 – 1.2 mol $mol^{-1}$), we observed an average inhibition to 65.6±3.6% activity compared to 100% control of autophosphorylated CaMKII in the presence of calmodulin. The strongest inhibition resulted in a CaMKII activity of only 51.1±3.1% at the PKC/CaMKII ratio of 3.0 mol $mol^{-1}$. We observed no PKC-dependent increase of the phosphorylation of the autonomy site of CaMKII probed with the antiactive CaMKII antibody (data not shown). Since CaMKII activity was only inhibited by PKC in the absence of calmodulin, calmodulin appears to protect CaMKII from transphosphorylation by PKC.

*Basal autophosphorylation inactivates gastric mucosal CaMKII phosphotransferase activity*

The inactivation of CaMKII by PKC transphosphorylation in the absence of calmodulin comprises aspects of CaMKII inactivation by basal autophosphorylation as reported for rat brain CaMKII (Hanson & Schulman, 1992; Colbran, 1993). As only limited information about the autoregulation of gastric mucosal CaMKII is available, we analyzed the CaMKII phosphotransferase activity either after calmodulin-activated autophosphorylation or after basal autophosphorylation, that is, in the absence of calmodulin, and compared its characteristics with our findings of the PKC-dependent inhibition of CaMKII activity.

In the absence of calmodulin, CaMKII only very moderately autophosphorylated at the autonomy site, as shown with an antiactive CaMKII antibody directed against the phosphorylated autonomy site (Figures 5a,b). In contrast to this, a clear time-dependent autophosphorylation of the autonomy site was detected, if calmodulin was present (Figures 5a,b). CaMKII became partially inactive for the phosphorylation of autocamtide-II after time-dependent basal autophosphorylation (Figure 5c). Autophosphorylation in the presence of calmodulin did not affect the CaMKII transphosphorylation activity (Figure 5c). Basal autophosphorylation between 10 and 20 min reduced CaMKII activity by 22 – 44% compared to activity without basal autophosphorylation. There is a notable similarity between the phosphorylation-dependent inhibition of CaMKII by PKC and by basal autophosphorylation: both inhibitions occurred only in the absence of calmodulin, and resulted in a linear decrease of CaMKII transphosphorylation activity (Figures 4, 5c).

## Discussion

In this study, we describe that both cholinergic-stimulated gastric acid secretion of rabbit parietal cells and CaMKII activity were attenuated by PKC-α. As recently described in carbachol-stimulated rabbit parietal cells, PKC-α translocated to the plasma membrane, and would, therefore, be expected to be activated (Fährmann *et al.*, 2002b). Stimulation-dependent translocation of PKC-α has also been observed in other mammalian tissues (reviewed in Kanashiro & Khalil, 1998). Calcium signals lead to a rapid and reversible translocation of calcium-dependent PKC isoforms to the plasma membrane. This is accompanied by an activation of the kinase activity. Subsequently, diacylglycerol increases the binding affinity of cPKC to the plasma membrane but does not retain cPKC at the plasma membrane after a decrease of the intracellular calcium (Oancea & Meyer, 1998).

In the last two decades, many physiological investigations were made to specify the role of PKC in gastric acid secretion (Anderson & Hanson, 1985; Muallem *et al.*, 1986; Beil *et al.*, 1987; Brown & Chew, 1987; Chiba *et al.,* 1989; Nandi *et al.*, 1996; Kopp & Pfeiffer, 2000; reviewed in Fährmann, 2000). Most of these reports indicate an attenuating rather than facilitating function of PKC in acetylcholine-mediated acid secretion at the level of parietal cells. Especially for the calcium-dependent PKC-α, an inhibitory role in parietal cells has been suggested (Nandi *et al.*, 1999; Fährmann *et al.*, 2002b). In contrast to this, CaMKII appears to play the role of an important mediator in cholinergic signal transduction for acid secretion. The nearly identical $IC_{50}$'s of KN-62 to inhibit carbachol-induced $H^+$ secretion of parietal cells as well as of purified CaMKII of parietal cell-containing gastric mucosa support the assumption of CaMKII as an important ruler in parietal cell function (Fährmann *et al.*, 2002a,b).

Treating parietal cells dose-dependently with Gö 6976 showed the attenuating effect of PKC-α on carbachol-induced acid secretion. In rabbit parietal cells Gö 6976 is assumed to be specific to PKC-α, since other calcium-dependent PKC isoenzymes were not detected by specific antibodies (Nandi *et al.,* 1996; Chew *et al.*, 1997). Selective inhibition of PKC-α by Gö 6976 increased carbachol-evoked $H^+$ release up to about one-third. The enhanced translocation of PKC-α in the presence of TPA in carbachol-stimulated rabbit parietal cells would be expected to enhance the phosphotransferase activity of PKC-α (Fährmann *et al.*, 2002b). Moreover, in the presence of TPA, carbachol-stimulated acid secretion was substantially inhibited down to basal acid release, presumably by full activation of PKC-α. The complete antagonization by Gö 6976 of the TPA-dependent inhibition of $H^+$ secretion revealed the suppressive action of PKC-α. Recently, it has been reported that Gö 6976 (100 nM) had no detectable effect on the carbachol-stimulated AP uptake of rabbit gastric glands (Chew *et al.*, 1997). Although comparing physiological data of parietal cells to gastric glands is only preliminary, we also observed that Gö 6976 at 100 nM had no significant effect on carbachol-induced $H^+$ secretion. However, Gö 6976 was most efficient at concentrations $>100$ nM. Excessive concentrations of both Gö 6976 and TPA resulted in an additional stimulation of carbachol-induced acid secretion, which indicates the involvement of at least another calcium-independent PKC isoform.

Another interesting finding was the inhibition of CaMKII activity in TPA-treated parietal cells. The concentration-dependent inhibition of both carbachol-induced acid secretion and cellular CaMKII activity by TPA remarkably correlate. The phorbolester TPA is not known to affect CaMKII activity directly, as CaMKII does not contain any site to bind phorbolester (Braun & Schulman, 1995). Since the inhibition by TPA was not additional to partially inhibited $H^+$ secretion by KN-62, this finding explicitly supports the model of a functional linking of PKC-α to CaMKII.

We obtained more insight into the interaction of PKC-α and CaMKII when we analyzed the action of PKC-α on CaMKII *in vitro* on the level of purified proteins. The high conservation of the catalytic as well as the regulatory domain of PKC-α in mammals (Kanashiro & Khalil, 1998) justifies the use of the rat brain isoform to study the action of PKC-α on gastric mucosal CaMKII. We found that a transphosphorylation of CaMKII *in vitro* occurred at an equimolar concentration of PKC and CaMKII resulting in the phosphorylation of at least one phosphorylation site in CaMKII. Although CaMKII appears to be a moderate substrate for PKC, this may be compensated, unusually, by similar intracellular concentrations of enzyme (PKC) and substrate (CaMKII) as indicated by immunoblot analysis (Fährmann *et al.*, 2002a,b). Thus, we assume that the moderate transphosphorylation of CaMKII by PKC seems to be sufficient to attenuate the CaMKII activity efficiently. Several data are consistent with a phosphorylation *in vivo.* Recently, a direct phosphorylation of rat brain CaMKII as well as related peptides by PKC was

reported (Waxham & Aronowski, 1993). However, the consequence for the CaMKII activity was not shown in this study. The consensus sequences for substrate recognition are similar to both PKC and CaMKII. Although there are some exceptions, PKC phosphorylates the motif RXXS/TXR whereas CaMKII prefers RXXS/T (Pearson & Kemp, 1991).

Moreover, we found similarities between the inhibition of CaMKII activity by transphosphorylation of PKC and by basal autophosphorylation of CaMKII. As it has recently been shown for CaMKII of rat forebrain, basal autophosphorylation occurred at the calmodulin-binding site (Hanson & Schulman, 1992; Colbran, 1993). This appears to interfere with the binding of $Ca^{2+}$/calmodulin, and, therefore, with the activation of CaMKII. Since the absence of calmodulin was a prerequisite to inhibit CaMKII by PKC, the transphosphorylation of PKC may occur at the calmodulin-binding site. Additional experiments are necessary to reveal the precise PKC phosphorylation site or sites in CaMKII. For CaMKII of rat forebrain, several autophosphorylation sites are known (Miller *et al.*, 1988; Dunkley, 1992; Hanson & Schulman, 1992), which may also be potential transphosphorylation sites (see Figure 6).

PKC action on CaMKII may interfere with the stepwise autoregulation of CaMKII. After a cholinergically mediated, rapid rise of intracellular calcium, the CaMKII holoenzyme has to undergo characteristic autophosphorylations to become autonomous and fully active. The oligomeric appearance of CaMKII is a prerequisite for autoregulation (Meyer *et al.*, 1992; Hanson *et al.*, 1994; Mukherji & Soderling, 1994; Brocke *et al.*, 1999). $Ca^{2+}$/calmodulin binding to two adjacent CaMKII subunits is required to activate one subunit, and to make a second subunit competent for the trans-subunit autophosphorylation at the autonomy site by the first subunit. The unique autoactivation mechanism of CaMKII needs several $Ca^{2+}$ spikes to become fully autonomous, which means autophosphorylation of all holoenzyme subunits, and is therefore time-consuming (Hanson *et al.*, 1994; Mukherji & Soderling, 1994; Brocke *et al.*, 1999; reviewed in Soderling *et al.*, 2001). The inhibitory transphosphorylation of CaMKII by PKC-α may compete with the activating autophosphorylation of CaMKII. The autonomy site of CaMKII does not seem to be identical to the PKC phosphorylation site. At least transphosphorylation by PKC-α retains holoenzyme CaMKII to only partial activity.

For gastric parietal cells, it is conceivable that PKC-α functions as an attenuator to save an inherent reserve to be activated by other secretagogues, or to limit $H^+$ release. Recently, it has been shown that the inhibitory effect of interleukin 1β on carbachol-stimulated acid secretion is mediated by PKC. The interleukin 1β-induced, local inhibition of acid secretion around gastric ulcers has been assumed to reduce further damage, and hence to support the healing mechanism (Beales & Calam, 1998, 2001). Our study suggests that PKC-α represents at least one possible PKC isoform in parietal cells to convey interleukin 1β induction to inhibit carbachol-stimulated acid secretion. Yet, more experimental work is needed to explain the precise function of PKC-α in acid secretion.

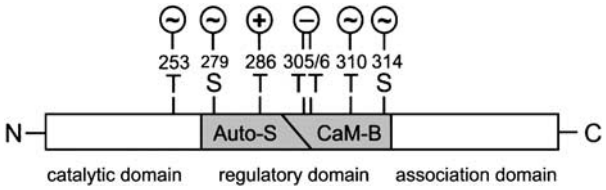


**Figure 6** Autophosphorylation sites of CaMKII. The scheme shows the three functional domains as well as the autophosphorylation sites of rat brain CaMKII-α (for details, see the 'Discussion' section) instead of gastric CaMKII, which is only partially sequenced. The effects of primary autophosphorylation are indicated: (+), a $Ca^{2+}$-dependent, primary autophosphorylation of CaMKII occurs at $Thr^{286}$ of the autonomy site (Auto-S) to become partially independent of $Ca^{2+}$/calmodulin for full activation of oligomeric CaMKII. (−), in contrast, a $Ca^{2+}$-independent primary phosphorylation of $Thr^{305/306}$ of the calmodulin-binding site (CaM-B) leads to an inhibited CaMKII. (∼), autophosphorylation sites at $Thr^{253}$, $Ser^{279}$, $Thr^{310}$, and $Ser^{314}$ are of uncertain function. Probably other autophosphorylation sites exist. Some autophosphorylation sites of CaMKII may represent putative transphosphorylation sites for distinct protein kinases. For gastric mucosal CaMKII, PKC-α appears to transphosphorylate at least one phosphorylation site resulting in an inhibited activity of CaMKII.

In summary, our results suggest that the calcium-dependent PKC-α is directly functionally linked to CaMKII in rabbit gastric parietal cells. Transphosphorylation of CaMKII by PKC-α results in a suppression of the phosphotransferase activity of CaMKII. This mechanism suggests being at least responsible for the attenuation of cholinergically induced acid secretion. The importance of this regulation for parietal cell signaling and attenuation of $H^+$ secretion is currently unknown. Combining our data with recent reports (Nandi *et al.*, 1999; Beales & Calam, 2001; Fährmann *et al.,* 2002a,b), it becomes more obvious that PKC-α plays a suppressive rather than obligatory role in the signal transduction of parietal cells.

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