3 Results

3.1. Demonstration of expression and phosphorylation of CRE-binding proteins in thyroid cells by Western blot analysis.

To test for expression of CRE-binding proteins in thyroid cells three commercially available antibodies were employed: (1) the anti-CREB polyclonal IgG antibody from UBI was raised in rabbits immunized with a synthetic peptide corresponding to amino acids 5- 21 of human CREB-327. It is claimed to show broad species reactivity, (2) the anti-phosphoCREB from UBI is produced by immunization of rabbits with a synthetic phosphopeptide corresponding to residues 123-136 of human CREB-327, and (3) the rabbit polyclonal IgG anti-CREM-1 was purchased from SantaCruz. It is produced by immunization with full-length polyhistidine tagged CREM-1 fusion protein, again of human origin. It is said to cross-react with mouse, rat and human protein.

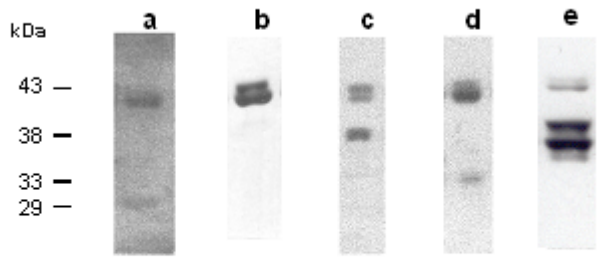

FIG.5 Western-blot analysis of CREB/M expression:
lanes a and b: detection of CREB and CREM protein, respectively,
in lysates of FTC133 carcinoma cells; lanes c-e: FTC133 (c),
FRTL5 (d), and RIN cells (e) tested with anti-posphoCREB.

Lanes *a* and *b* of Fig. 5 show typical results obtained with anti-CREB and anti-CREM-1, respectively, when whole protein lysates of the human follicular carcinoma cell line FTC 133 were blotted. Employment of nuclear lysates gave comparable results (not shown). Both antibodies detected the expected doublet band at about 43-kDa, corresponding to CREB-327 and CREM-τ isoforms, respectively. In addition, anti-CREB detected a minor band of approximately 29- kDa, which sometimes was also identified as a faint band on anti-phosphoCREB blots (not visible in Fig. 5). The identity of this minor CREB-form is not clear, but a good candidate is CREB-W. CREB-W is generated by inclusion of alternatively spliced exon W into the CREB transcript resulting in the premature stop of translation and subsequent reinitiation at two downstream internal methionine codons (Girardet et al., 1996). As the UBI CREB-antibody is directed against an amino-terminal stretch, the two internally translated truncated CREB

isoforms I-CREB(I) and I-CREB(s) (8- and 16- kDa, respectively) could not be detected (see also Fig. 6).

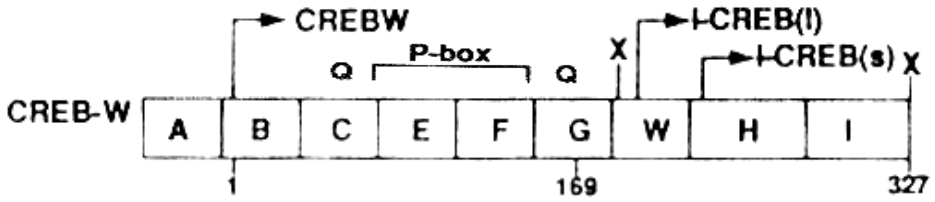

FIG. 6 Diagrammatic depiction of the CREB-W mRNA resulting from alternative exon splicing. Modified from Girardet C., Walker W.H., and Habener J.F., 1996.

Alternatively, the 29- kDa band may represent CREM- isoforms α, β, and γ (see Introduction Fig. 3), which also migrate at 29- kDa. These repressors were previously reported to be expressed in dog thyroid tissue (Uyttersprot and Miot, 1997). However, the band was not detected by the anti- CREM-1 antibody, which would be expected to show broad cross-reactivity with other CREM isoforms.

The 43- kDa doublet was also recognized by the phosphoCREB antibody (lanes *c* and *d*). In addition, in FTC 133 cells a second, equally strong band was found with an apparent molecular weight of 38- kDa, which was not present in FRTL-5 cell lysates. Further, the band was neither detected by the CREB nor the CREM-1 antibody. According to the manufacturer of the phosphoCREB antibody, and in agreement with the literature, this band corresponds to phosphoATF-1. ATF-1 and CREB are clearly distinct proteins but have a 75% amino acid similarity. In particular, ATF-1 shows extensive homology in the P-box area. The phosphoCREB antibody was derived against a synthetic peptide with the sequence KRREILSRRPpSYRK corresponding to CREB-327 amino acids 123- 136, the corresponding amino acid sequence of ATF-1 is KAHGILARRPpSYRK. In contrast, the amino-terminal stretch against which anti-CREB is directed (SGADNQQSGDAAVTEA in CREB) is not conserved in ATF-1 (S.....HKSTTSETA). Thus it is well-conceivable that phosphoATF-1 is recognized by anti-phosphoCREB, but that ATF-1 is neither recognized by anti-CREB nor anti-CREM-1.

Finally, in lane *e* the pattern of P-box comprising proteins in RIN cells, a rat insulinoma cell line, is shown for comparison. The bands are clearly distinct from those in thyroid cells.

In summary, it can be stated that CRE-binding proteins are expressed and phosphorylated in thyroid cells in a tissue- and species- specific manner, with CREB-327 and CREM-τ isoforms found in both, rat and human cells, while ATF-1 being additionally expressed in cells of human origin.

3.2. TSH induces CRE-binding protein phosphorylation in a dosage- and time- dependent manner.

Direct demonstration of thyroid CRE-binding protein phosphorylation in response to TSH-stimulation was first achieved by a cooperation of the groups of M. Montminy and J. Meinkoth (Armstrong et al., 1995), who employed a phospho-serine 133 specific antibody to examine CREB-phosphorylation in FRTL-5 cells. However, FRTL-5 cells do represent an immortalized cell line, in addition from a rodent species, and results observed in these cells can´t be extrapolated to human tissue without further testing. Hence, the effects of TSH on human thyrocytes in primary tissue culture were compared with those on FRTL-5 cells.

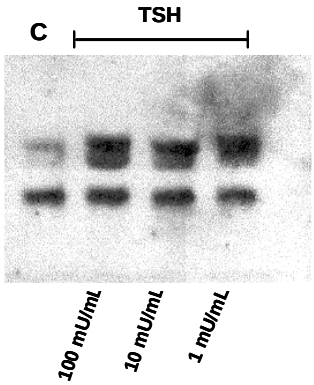

FIG.7 Concentration-dependent CREB/M-phosphorylation upon stimulation with TSH in human thyroid primary cells.

As shown in Fig. 7, TSH at concentrations of 1, 10 or 100 mU/mL induced a strong increase in CRE-binding protein phosphorylation after 30 min of exposure. Moreover, the response is concentration-dependent (compare also Fig. 16). Examining FRTL-5 cells R. Armstrong *et al.* were able to distinguish three phases in the response elicited by stimulants of the cAMP pathway, that is an early induction of phosphorylation reaching its maximum between 15 and 30

min (Hagiwara et al., 1993), followed by a subsequent attenuation phase during which dephosphorylation through protein phosphatase PP-1 balanced CREB-phosphorylation, and, finally, upon prolonged stimulation a refractory period was observed, caused by a five-fold, selective downregulation in PKA catalytic subunit. Similar findings are obtained when the kinetics of TSH-induced CRE-binding protein phosphorylation in human primary thyroid cells are examined. Figs.8 and 9 show anti-phosphoCREB blots of cell lysates during 4 hrs and 24 hrs-stimulation periods, respectively.

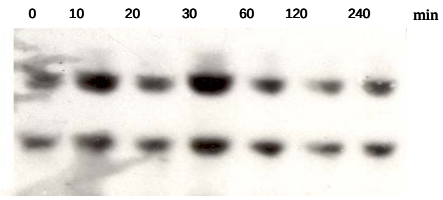

FIG.8 Kinetics of CREB/M-phosphorylation during TSH-stimulation of human thyrocytes in primary cell culture.

FIG.9 Attenuation of CREB/M-phosphorylation during prolonged challenge of primary thyroid cells with TSH.

Here, too, a swift increase in phosphorylation is observed after 10 min, declining to basal levels after about 2 hrs, until at about 12 hrs phosphorylation falls beyond the basal level, indicating overstimulation. Interestingly however, in addition a transient decrease at about 20 min was observed repeatedly, possibly pointing to a biphasic phosphorylation response. This was never seen in TSH-stimulated FRTL-5 cells (Fig. 10 *a*), however, it was sometimes seen when adenylate cyclase was stimulated directly with forskolin (Fig. 10 *b*).

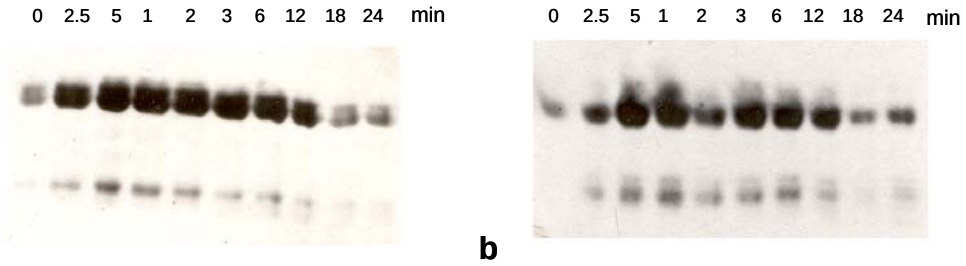

FIG.10 CREB/M-phosphorylation in rat FRTL5 cells stimulated with either 10 mU/mL TSH (a), or 10 uM forskolin (b), respectively.

Hence, human thyrocytes do respond to TSH stimulation with a strong and concentration-dependent CRE-binding protein phosphorylation. The three phases described previously in FRTL-5 cells are also noted in human cells, however, it appears that the response is biphasic with maximal peaks at 10 and 30 min.

3.3 Dosage- and time-dependent phosphorylation upon stimulation with tyrosine kinase receptor agonists EGF and insulin.

The P-box appears to harbour recognition sites for almost any protein kinase of importance described so far, and accordingly, many signalling cascades have meanwhile been shown to end in CREB/M-phosphorylation. Hence, it seemed likely that other factors than TSH might induce CREB/M-phosphorylation in the thyroid.

Here two well-known growth factors with opposing effects on differentiation functions, epidermal growth factor (EGF) and insulin, were examined for their potential in CREB/M-phosphorylation induction. Human thyrocytes in primary tissue culture were tested with either EGF at concentrations of 0.1, 1 or 10 ng/mL or insulin at concentrations of 100 ng/mL, 500 ng/mL, 1 µg/mL or 10µg/mL. Exposure time again was 30 min. As depicted in Fig. 11, both tyrosine kinase ligands were found to be inductors of CRE-binding protein phosphorylation, and the response was also concentration dependent. The maximal EGF effect was elicited at 1 ng/mL, in contrast, responses to insulin required dosages in the µg/mL range, indicating the employment of IGF- rather than insulin- receptors.

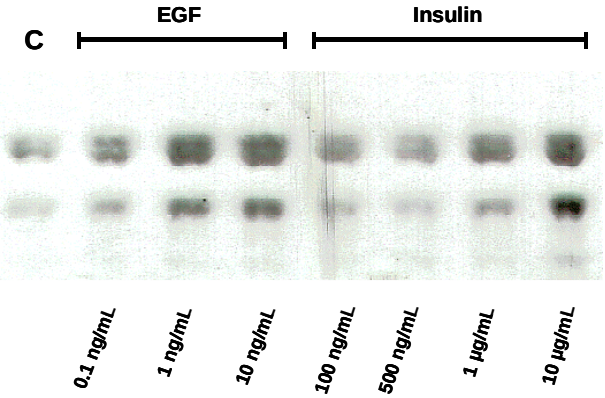

FIG.11 Dosage-dependent CREB/M- phosphorylation upon stimulation with tyrosine receptor agonists EGF and insulin in thyroid primary cells.

In Fig. 12 the kinetics of tyrosine kinase receptor mediated CREB/M phosphorylation are shown.

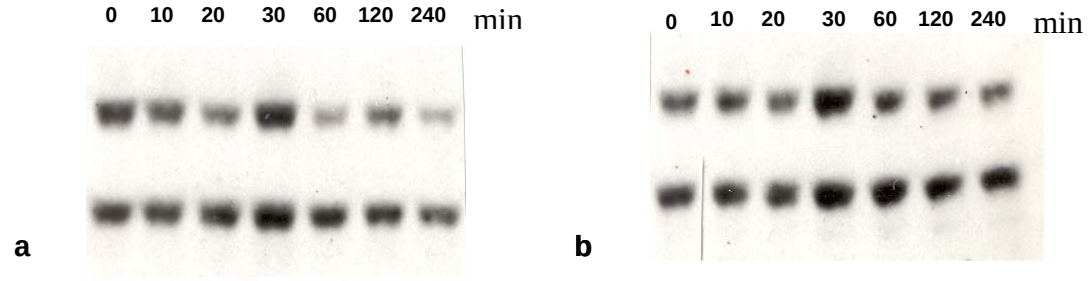

FIG.12 Human thyroid primary cells were stimulated with either 1 ng/mL EGF (a) or 10 µg/mL insulin (b) for the times indicated and subsequently blotted with anti-phospho CREB.

In human primary thyrocytes the effects induced by EGF and insulin were short, with a maximum at 30 min found for EGF, and at either 10 or 30 min in the case of insulin- stimulation. On the whole, reactions elicited by insulin were found rather variable (compare for instance Fig. 14).

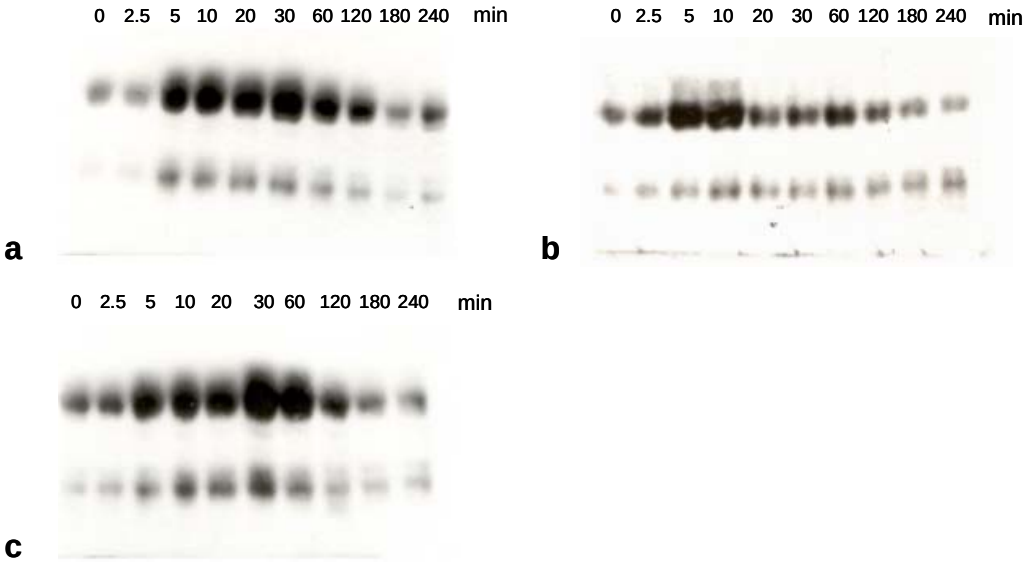

FIG.13 CREB/M-phosphorylation in rat FRTL5 cells stimulated with either 5 ng/mL EGF (a), 10 µg/mL insulin (b), or 100 nM TPA (c).

Finally, Fig. 13 again displays results obtained in FRTL- 5 cells. In these cells EGF induced responses comparable in strength to those elicited by TSH, forskolin, or direct activation PKC

by TPA. In contrast, the response to insulin was clearly weaker, falling to basal levels after about 20 to 30 min.

3.4. Comparison of CRE- binding protein phosphorylation patterns in thyroid primary cells with those in thyroid carcinoma cell lines.

Disturbances in growth factor responses are a hallmark of malignant tumours, and the thyroid is no exception to this rule. Therefore growth factor induced CREB/M- phosphorylation in four human thyroid carcinoma cell lines, two follicular and two anaplastic, were compared to the responses of human thyrocytes isolated from paranodular goitre tissue.

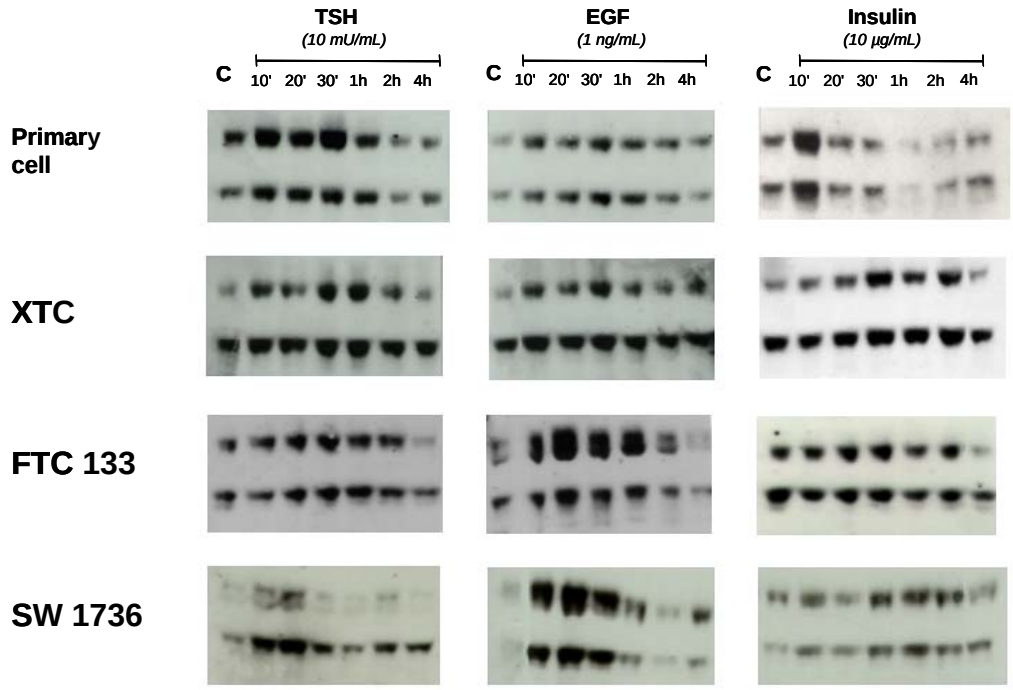

FIG.14 Patters of growth factor-induced CREB/M-phosphorylation in human thyroid primary cells as compared to those in various thyroid carcinoma cell lines.

Typical results obtained with primary cells are depicted in the first row of Fig. 14. The most pronounced effect in these cells was always induced by TSH, while EGF elicited only a minor response. In contrast, reactions to insulin were highly variable, the example shown in Fig. 14 representing a strong responder. In the rows below, the behaviour of XTC, FTC 133, and

SW1736 cell lines are shown. The XTC cells are unusual in that they were derived from a Hürthle cell carcinoma, a commonly well-differentiated subtype of the follicular thyroid carcinoma, whose particular malignancy is due to early hematogenic metastasis formation. In these cells responses to TSH, EGF, and insulin were almost indistinguishable.

In the rows below, the behaviour of XTC, FTC 133, and SW1736 cell lines are shown. The XTC cells are unusual in that they were derived from a Hürthle cell carcinoma, a commonly well-differentiated subtype of the follicular thyroid carcinoma, whose particular malignancy is due to early hematogenic metastasis formation. In these cells responses to TSH, EGF, and insulin were almost indistinguishable. The next row shows results gained with FTC 133 cells. These cells were cloned from a mediastinal lymph node metastasis of a patient with a poorly differentiated follicular carcinoma (Goretzki et al., 1990). They present a well established cell line, and were previously found to express functional TSH-receptor, albeit in reduced numbers. Accordingly, the TSH-stimulated increase in CREB/M- phosphorylation was significantly lower than in primary cells. In contrast, the response to EGF was strongly elevated in FTC 133 cells. The last row shows results obtained with SW1736 cells, an anaplastic carcinoma cell line. In these cells no reproducible effects were seen upon TSH- stimulation, the weak response shown here most likely being due to contaminations in the TSH preparation as this is gained from bovine pituitary gland. Reactions to EGF again were abnormally intensive, being even higher than in FTC 133 cells. Concerning insulin, there appears to be a general tendency of insulin sensitivity to parallel responsiveness to TSH.

Finally, in the second anaplastic carcinoma cell line examined here, the HTh 74 cells, a distinct autocrine stimulation of CREB/M- phosphorylation was encountered. In these cells alone the exchange of the medium induced a strong round of phosphorylation (see Fig. 15 a).

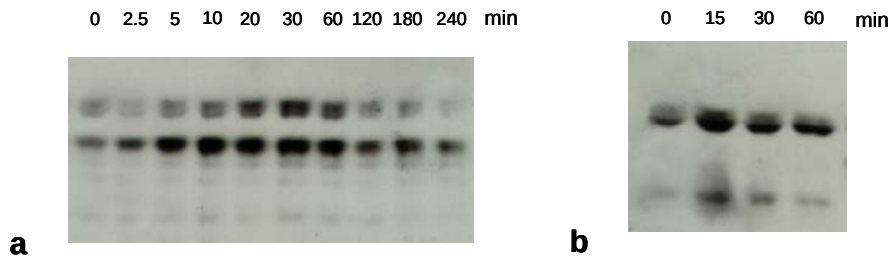

FIG.15 Induction of CREB/M-phosphorylation in Hth 74 anaplastic carcinoma cells by an apparently autocrine mechanism (a), and (b), CREB/M-phosphorylation in rat FRTL5 cells after exposure to medium preconditioned through Hth 74 cultures.

Since HTh 74 cells are known to carry PDGF- receptors, a receptor type normally restricted to cells of mesenchymal origin (Heldin et al., 1991), the preconditioned, filtered medium was offered to FRTL-5 cells. As shown in Fig. 15 b FRTL-5 cells reacted with CREB/M-phosphorylation to exposure of HTh 74 conditioned medium. Hence, the stimulatory factor appears to be secreted and to bind a receptor type normally present in the thyroid.

Taken together, patterns of CRE- binding protein phosphorylation are clearly aberrant in thyroid carcinoma cells: while the responsiveness to TSH and insulin appears to decline with increasing dedifferentiation, sensitivity to the dedifferentiative growth factor EGF opposingly increases. In addition, at least in HTh 74 cells, there is evidence for an autocrine loop inducing strong CREB/M- phosphorylation.

3.5. CREB/M- phosphorylation in „cold“ and „hot“ nodules.

Heterogeneous expression of tyrosine kinase receptors and dysregulation of autocrine loops, in this case involving particularly IGF-1 (Williams et al., 1989), have also been suspected of playing an important role in the pathogenesis of benign nodular transformation of the thyroid gland (see, for instance, Studer and Derwahl, 1995).

Here a small number of „hot“ (n = 5) and „cold“ (n = 3) nodules was tested for their responses to TSH, and provided sufficient tumour material was available, to insulin and EGF. At least one autonomous adenoma (Fig. 16) was detected even in this small series that displayed clearly abnormal behaviour.

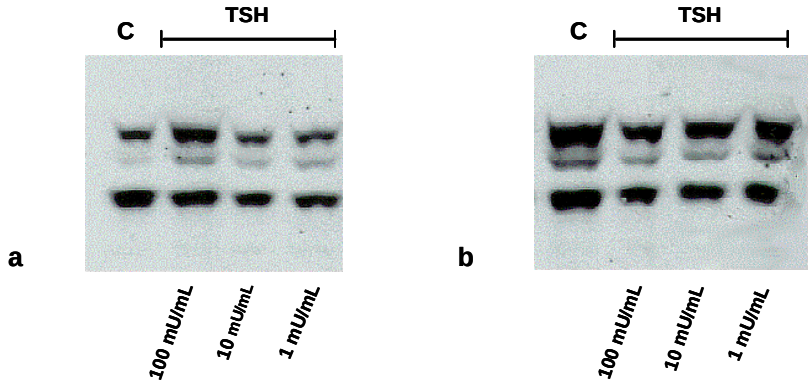

FIG.16 Overstimulation of CREB/M-phosphorylation by TSH found in primary culture of thyroid cells from an autonomous adenoma (b), in (a) paranodular tissue from the same patient is shown as comparison.

Compared to cells isolated from the paranodular tissue, these primary adenoma cells showed elevated basal CREB/M- phosphorylation levels. Moreover, when further stimulated with TSH, a concentration- dependent decrease in phosphorylation was observed. In contrast, the paranodular cells reacted in the expected way described above.

In summary, this preliminary data certainly encourage investigations on a larger scale.

3.6. Study of pathways employed in CREB/M- phosphorylation with various kinase inhibitors.

To investigate the signal cascades leading to phospho- CREB/M formation, specific protein kinase inhibitors for the main four protein kinases previously shown to phosphorylate CRE-binding proteins, i.e. PKA, PKC, p70S6K, and Camk II, were applied.

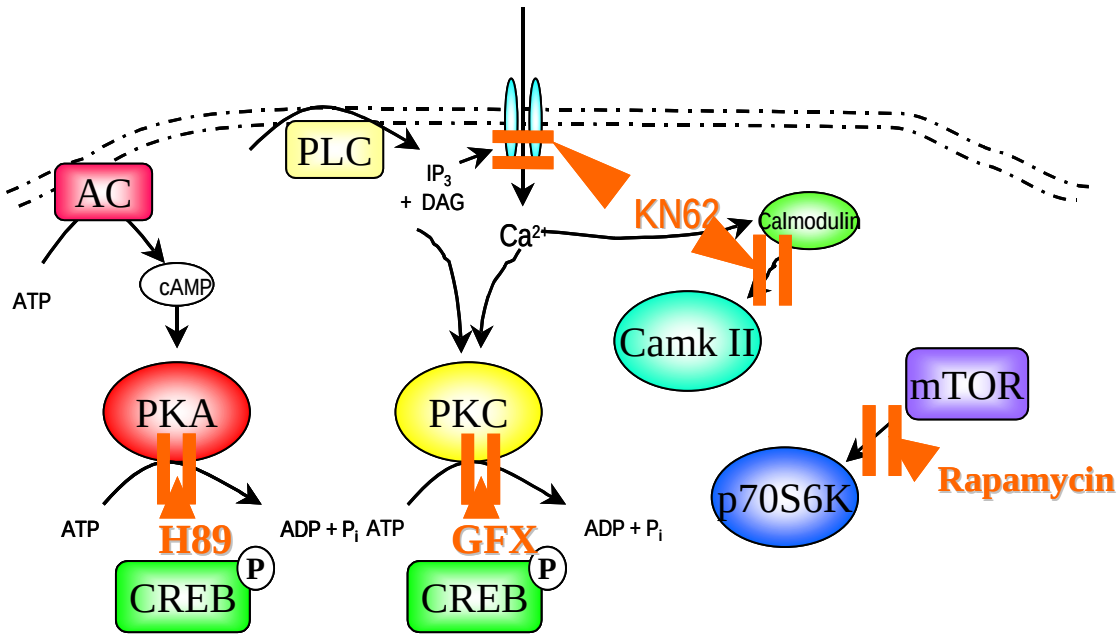

FIG.17 Illustration of protein kinase inhibitor targets.

he PKA- inhibitor H89 and the PKC- inhibitor GF109203X are both derivatives of staurosporine, and act by competitive binding to the ATP- binding site of protein kinases. H89

was previously demonstrated to be specific for PKA- inhibition up to concentrations of 30 µM (Chijiwa et al., 1990), and was used here at 20 µM. GFX was reported to show some concentration- dependent PKC- isotype specificity (Martiny- Baron et al., 1993). The concentration of 1.6 µM applied here would thus be expected to inhibit classic as well as novel isoforms, but not atypical PKCs and the related PKN.

Rapamycin blocks p70S6K indirectly by preventing the association between activated p70S6K and the positive regulator mTOR. It was applied at 20 nM. Finally, KN62 inactivates Camk II by interference with calmodulin binding. However, KN62 was also reported to affect PKC activity through blockage of Ca^{2+}- channels (Li et al., 1992). Here it was used at 15 µM.

The respective points of interference for these four inhibitors are summarized in Fig. 17.

3.6.1. Influence of kinase inhibitors on TSH- and insulin-dependent phosphorylation.

As the TSH- induced response appeared to be biphasic, inhibition was attempted at 10 min (first peak of CREB/M- phosphorylation), 30 min (second peak), and 45 min of stimulation. Only H89 and GFX were found to have a relevant, reproducible effect throughout this whole interval (see left side of Fig. 18).

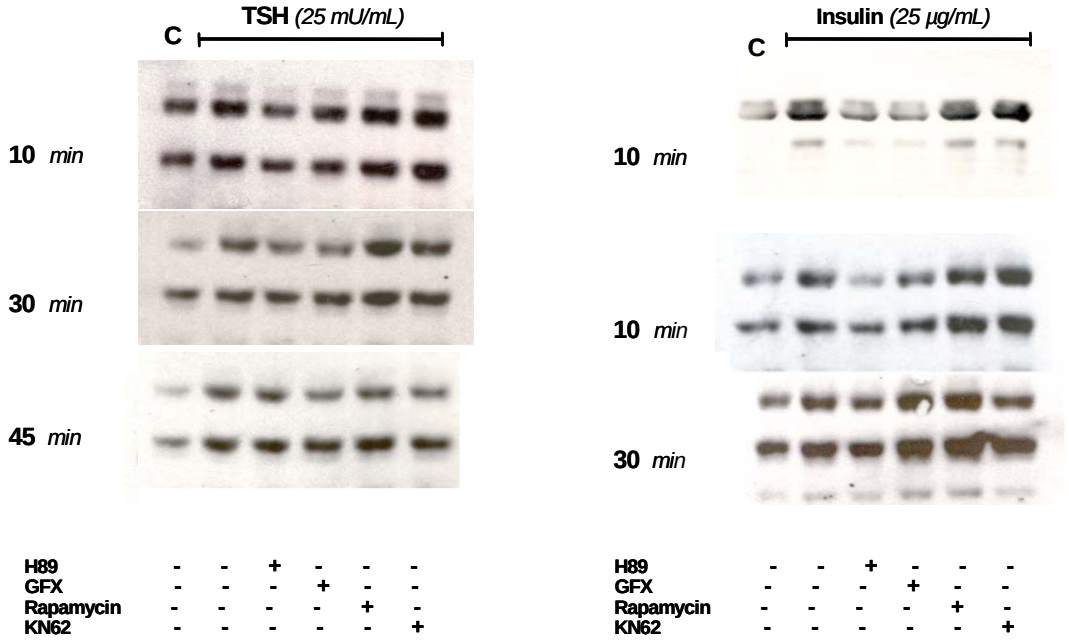

FIG.18 Influence of various protein kinase inhibitors on TSH- and insulin- stimulated CREB/M-phosphorylation, respectively.

However, at no time point was complete inhibition of CREB/M- phosphorylation affected by either of them. This was only achieved when both were applied together (not shown). Moreover the relative contributions made by H89 and GFX varied in a time- dependent manner: thus, at 10 min of TSH- stimulation the effect of H89 dominated, then at 30 min H89 and GFX effects became comparable, while at 45 min inhibition by GFX was stronger.

On the right side of Fig. 18 inhibitor effects observed during insulin stimulation are shown. Here, too, of the inhibitors examined only H89 and GFX had an effect on CREB/M- phosphorylation. But in contrast to the results obtained under TSH- stimulation, the relative contributions made by these two inhibitors appeared to vary from experiment to experiment (compare, for instance, first and second row of Fig. 18) rather than in a time- dependent manner. Thus, the pattern of inhibition within one experiment was maintained, no matter wether values were taken at 10 or 30 min of insulin- stimulation (see lower part of Fig. 18).

In summary, the experiments with protein kinase inhibitors thus confirm the suspected biphasic nature of the response to TSH: there appear to exist two independent cascades induced through TSH, one employing PKA and preceding the second pathway, which proceeds via a classic or novel PKC. Also, the results obtained with insulin again are rather variable, but, too, always involved PKA and PKC activation.

3. 6. 2. Influence of kinase inhibitors on EGF- and TPA- induced phosphorylation.

In Fig. 19 the influence of protein kinase inhibitors on 30 min of EGF- or TPA- stimulation is shown.

In both cases, the phosphorylation response could be blocked completely by pretreatment of cells with either H89 or GFX, while rapamycin and KN62 were ineffective. Consequently, it appears that the EGF-induced CREB/M- phosphorylation involves the consecutive action of PKC and PKA within a common pathway. Moreover, as TPA was able to mimic the EGF effect, it is likely that the activation of a classic or novel PKC isoform preceds the subsequent PKA-activation.

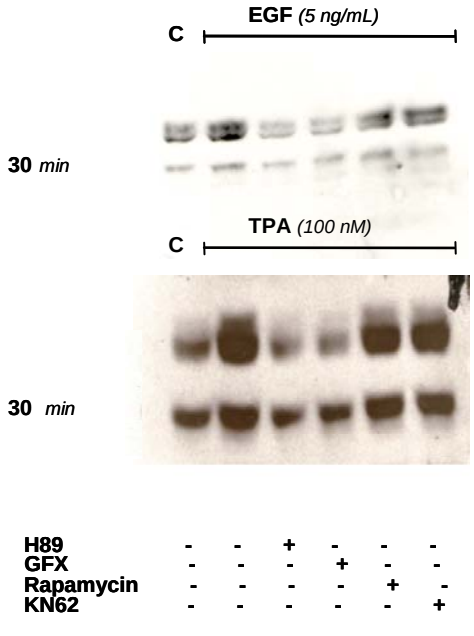

FIG.19 Influence of various protein kinase inhibitors on EGF- and TPA- stimulated CREB/M-phosphorylation, respectively.

3. 7. ICER- expression in thyrocytes.

Because transcriptional control of the negative feedback effector „ICER“ is exerted through four CRE- sites within the CREM P_2- promoter, ICER expression may conveniently be followed as a kind of „endogenous reporter“ of CREB/M- activity. Employing semiquantitative RT-PCR, a slight, but reproducible induction was detected upon stimulation with all three growth factors tested (i.e. TSH, insulin, and EGF). This induced expression was maximal at around 2 hrs of growth factor exposure in FRTL-5 cells (compare Fig. 20), and at around 4 hrs in human primary thyrocytes (data not shown). However, these results are in contradiction to findings reported by N. Uyttersprot *et al.* for dog primary thyrocytes, where RNase protection assays revealed an increase in ICER transcription only upon stimulation by TSH or forskolin, but not by either EGF or TPA (Uyttersprot et al., 1999). As unspecific effects of growth factor stimulation on transcription or RNA stability cannot be ruled out, reporter plasmid studies were carried out to further address this question.

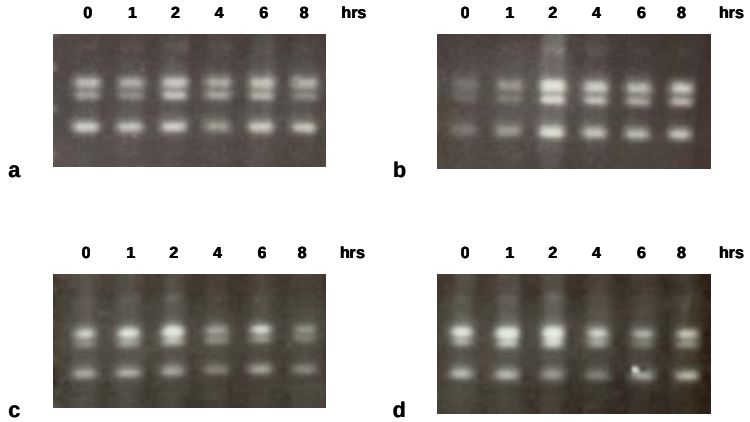

FIG.20 Kinetics of ICER-induction in FRTL5 cells by either (a) 10 mU/mL TSH, (b) 10 µM forskolin, (c) 1 ng/mL EGF or (d) 10 µg/mL insulin, respectively

3. 8. Transient transfection studies with the CRE- reporter plasmid 4xSCE1/2T81 in HTC- TSHr cells.

For reporter assay experiments the plasmid 4xSCE1/2T81 was used, which was kindly provided by W. Knepel (Göttingen). The basis of this construct is the luciferase shuttle vector pT81luc. pT81luc is one of a series of vectors designed by S. K. Nordeen for assaying promoter activity in mammalian cells. It harbours the high copy number replicon from pMB1 and an ampR marker for propagation and selection in *E. coli*, and the firefly luciferase gene transcribed from bp -81 to +52 of the herpes simplex thymidine kinase promoter as a reporter gene with minimal basal activity. From this, 4xSCE1/2T81 was generated by insertion of a synthetic oligonucleotide comprising four copies of the somatostatin CRE consensus sequence into a *BamHI* site just upstream of the HSV tk promoter (Oetjen et al., 1994).

Along with the CRE- reporter plasmid a fixed amount of a ß- galactosidase reporter was cotransfected: in the control plasmid pSV ß- Gal, expression of ß- galactosidase is under control of the very strong SV40 early promoter, thereby granting high levels of CRE- independent expression of the reporter enzyme. In this way, luciferase measurements can be corrected for differences in transfection efficiency as well as for unspecific effects due to growth factor influences on transcription, translation or RNA and protein stability.

Thyroid cells were previously shown to exhibit an absolute refractory phase of several days after TSH exposure (Armstrong et al., 1995), consequently transient transfection had to be carried out in the absence of TSH. Under these circumstances only HTC- TSHr cells were found to give satisfactory transfection rates with 4xSCE1/2T81. The HTC cell line represents a subclone of the

FTC 133 cell line, which has lost endogenous TSH receptor expression. This was substituted for by stable transfection with the human TSH receptor cDNA (Derwahl et al., 1993).

3. 8. 1. Induction of CRE- controlled luciferase by forskolin and TPA.

Transfected HTC- TSHr cells were kept in serum- reduced medium (0.5% w/v FCS) for one day prior to stimulation with either 10 µM forskolin or 100 nM TPA. Results summarized in Fig. 21 are the means +/- SD of two independent experiments with values taken in duplicate. As CRE-regulated gene activity was previously shown to be required throughout G1 up to the G1-S boundary (Desdouets et al., 1995), a correspondingly long observation period of 24 hrs was chosen.

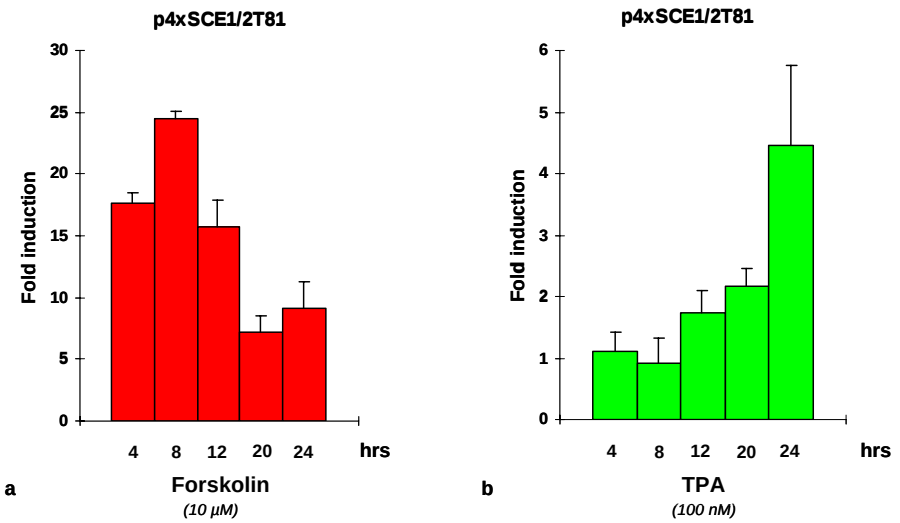

FIG.21 Induction of the CRE-reporter plasmid p4xSCE1/2T81 in transiently transfected HTC-TSHr cells by either forskolin (a) or TPA (b), respectively.

As expected, direct activation of adenylate cyclase by forskolin resulted in a fast and pronounced increase in reporter activity: induction reached its maximum at 8 hrs of stimulation with an increase of about 25fold over basal activity. Previous studies examining CRE- reporter induction in other cell types failed to notice a TPA effect. However, these studies were limited to a maximal 12 hrs observation period (Brindle et al., 1995; Lee et al., 1990). In the HTC- TSHr system used here also no significant effect of TPA was observed until at about 20 hrs of stimulation, with an approximately 4.5fold increase of luciferase activity at 24 hrs. The effect appeared to be specific, as no such rise in reporter activity was detected in insulin stimulated

cells (data not shown). The cause for the induction delay is not known. It may indicate an indirect effect of TPA requiring the induction of a positive regulator, or else the downregulation of some inhibitor may be necessary. As prolonged TPA treatment is known to cause downregulation of classic and novel PKCs, the requirement for release of an inhibitory effect by such a PKC isoform would be the most obvious explanation.

3. 8. 2. Induction of CRE- controlled luciferase activity by TSH and EGF.

In Fig. 22 the effects of TSH (10 mU/mL) and EGF (10 ng/mL) on luciferase activity are shown. There is a striking similarity in the kinetics of reporter induction by TSH and forskolin on the one hand, and EGF and TPA on the other: like observed for forskolin, TSH simulated luciferase activity with an early maximum at 8 hrs (approximately 5fold induction), while EGF displayed a delayed effect with an approx. 2.5fold increase after 24 hrs. Interestingly, when comparing the results to those of Uyttersprot *et al*., there is also an increase in ICER levels after EGF treatment for 24 hrs visible on the RNase protection gel presented. However, as the authors state that „...this result was not reproduced in other experiments“ (Uyttersprot et al., 1999) the significance has to remain unclear.

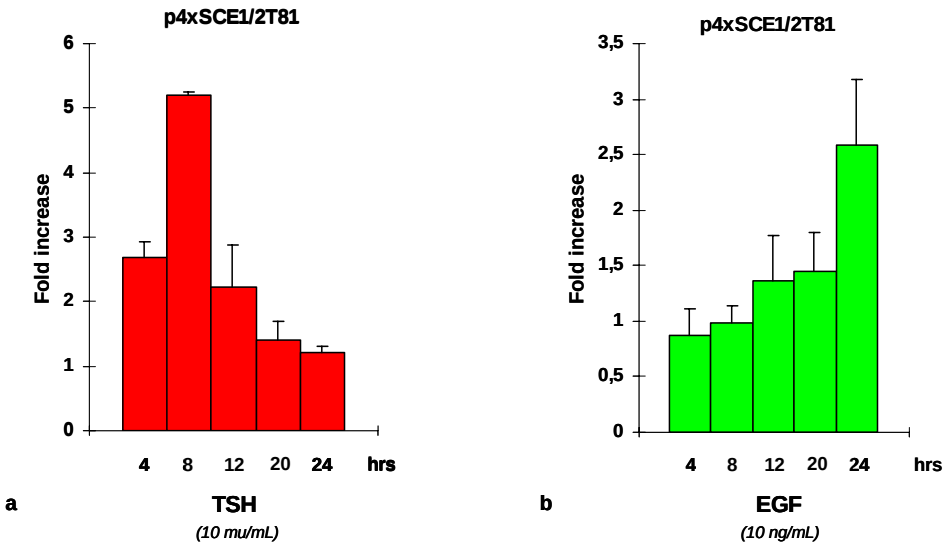

FIG.22 Induction of the CRE-reporter plasmid p4xSCE1/2T81 in transiently transfected HTC-TSHr cells by either TSH (a) or EGF (b), respectively.

3. 9. Expression of conventional and novel PKC isoforms in HTC- TSHr cells.

Prolonged treatment with phorbolesters commonly leads to downregulation of susceptible PKC-isoforms, and this is also seen in the thyroid (Wang et al., 1995). To test wether the downregulation of some PKC isoform may coincide with the delayed induction observed in CRE- reporter assays, whole cell lysates of stimulated HTC- TSHr cells were blotted with PKC isoform- specific antibodies at 0, 4, 8, 12, 20, and 24 hrs of stimulation. For the thyroid, expression of PKC isoforms α, β, δ, ε, and ζ has been described previously (Feliers and Pavlovic- Hourner, 1994). However, since atypical PKCζ is not induced by TPA, it was not examined here.

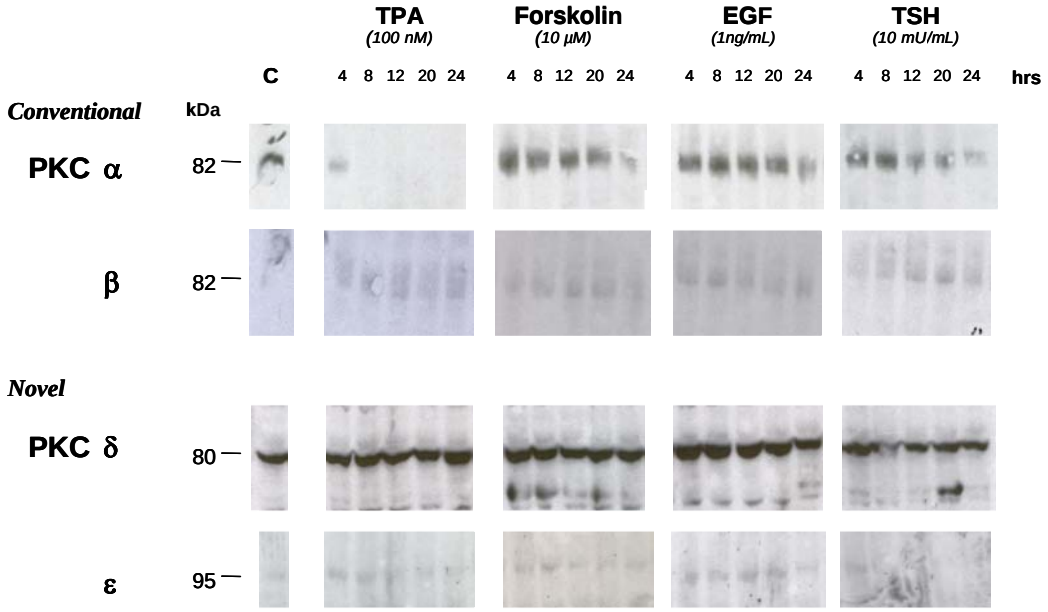

FIG.23 Basal expression of various PKC isoforms in HTC-TSHr cells and influence of prolonged stimulation with the agents indicated on levels of PKC α, β, δ, and ε isoforms, respectively.

As shown in Fig. 23, unstimulated HTC- TSHr cells expressed PKCα and δ at comparatively high levels, while PKCβ and ε were hardly detectable. This is somewhat surprising, because PKCδ expression was previously found to be TSH- dependent (Matowe W. C. et al., 1996). Moreover, PKCδ levels remained constant during the 24 hrs stimulation period with either TPA

(100 nM), forskolin (10 µM), EGF (1 ng/mL), or TSH (10 mU/mL). This, too, is in contradiction to previous observations made in FRTL-5 cells, where 300 nM TPA resulted in the fast downregulation of PKCα, δ, and ε (Wang et al., 1995). In contrast to this, PKCα was downregulated by all factors tested. However, the effect of TPA was clearly the most pronounced, causing a decrease in PKCα below the detection limit within 8 hrs of exposure.

Taken together, it thus appears that there is no correlation between the down- or upregulation of some PKC isoform with the late induction phase of CRE- controlled gene expression during TPA and EGF treatment. Beside this, an aberrant expression pattern of PKCδ was discovered in HTC-TSHr cells. Whether this may be connected to the EGF- hyperresponsiveness observed in FTC 133 and HTC cells is currently not clear, however, PKCδ was previously described as a member of the EGF- induced signal cascade (see, for instance, Dennings et al., 1996).