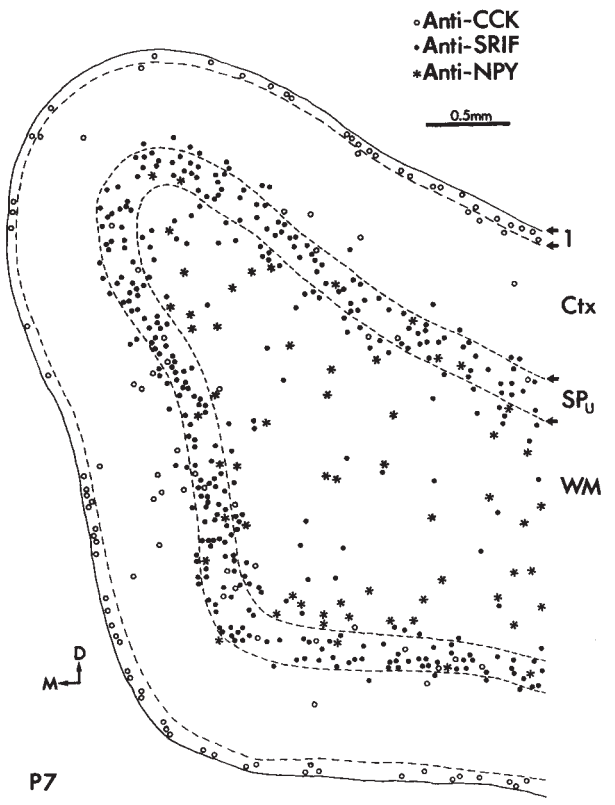

Fig. 4 The peptide immunoreactivity of subplate cells varies with their tangential position. Three adjacent 80-μm-thick coronal sections through the visual cortex of a P7 cat were immunostained with one of the antisera against CCK, NPY or SRIF. Each section was drawn with a camera lucida attachment and the location within the telencephalon of all labelled cells with a given peptide immunoreactivity was marked. The three drawings were then superimposed. Symbols identify cells immunoreactive for a given peptide: open circles, CCK; closed circles, SRIF; asterisks, NPY. Note the exclusive presence of CCK immunostained cells in layer 1, the clustering of SRIF immunostained cells in the upper subplate (SP_U), and the NPY immunostained cells deep in the white matter (WM). Ctx, cortex; M, medial; D, dorsal.

by two recent observations. Firstly, MAP2-immunoreactive cells thought to be Cajal–Retzius cells have been found in the marginal zone of mice embryos[30] and secondly in the telencephalon of human fetuses, some cells located in the subplate are also immunoreactive for SRIF[31].

The subplate cells are neurons that receive synaptic inputs during development. The most likely sources of the presynaptic inputs are the ingrowing thalamocortical and corticocortical axons, which accumulate in the subplate when the subplate cells are present[3–6,32,33]. Thus, the subplate cells may serve as the temporary targets of ingrowing afferent systems. If so, these cellular elements could represent part of an early and transient neural circuit that precedes the establishment of adult cortical connectivity. In addition, the distinct tangential domains occupied by cells of a given neuropeptide immunoreactivity suggest that different groups of subplate cells could serve distinct functions during the development of the mammalian cerebral cortex.

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A glucagon fragment is responsible for the inhibition of the liver Ca^{2+} pump by glucagon

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Glucagon specifically inhibits the Ca^{2+} pump in liver plasma membranes independently of adenylate cyclase activation[1]. However, this inhibition is only observed at high concentrations of glucagon ($K_i = 0.7$ μM). Moreover, in the presence of bacitracin, an inhibitor of glucagon degradation[2], the Ca^{2+} pump is no longer sensitive to glucagon[3]. These findings suggest that a fragment of glucagon might be the true effector of the liver Ca^{2+} pump. Pairs of basic amino acids are recognized as potential cleavage sites in post-translational processing of peptide hormones[4–6]. The glucagon molecule includes a dibasic doublet (Arg 17–Arg 18). Therefore, we have examined the action of glucagon(19–29) on the liver Ca^{2+} pump. This peptide was obtained from glucagon by tryptic cleavage and separated by reverse-phase high-performance liquid chromatography. We found that glucagon(19–29), which is totally ineffective in activating adenylate cyclase, inhibited both the Ca^{2+}-activated and Mg^{2+}-dependent ATPase activity ((Ca^{2+}-Mg^{2+}) ATPase) and Ca^{2+} transport in liver plasma membranes with an efficiency 1,000-fold higher than that of glucagon. Glucagon(1–21) was completely inactive; glucagon(18–29) and glucagon(22–29) acted only as partial agonists of glucagon(19–29). These results indicate that glucagon(19–29), obtained by proteolytic cleavage of glucagon, is likely to be the active peptide involved in the inhibition of the liver Ca^{2+} pump. We suggest that glucagon may be a precursor of at least one biologically active peptide.

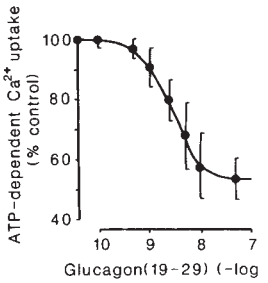

Fig. 1 Inhibition by glucagon(19-29) of the ATP-dependent Ca^{2+} uptake by liver plasma membrane vesicles. Results are the mean of five different experiments, done with five different plasma membrane vesicle preparations; bars, ±s.e.m.

Methods. Plasma membrane vesicles were prepared from liver of female albino Wistar rats (100-120 g body weight) according to Prpic *et al.*[7], using a Percoll gradient. Glucagon(19-29) was prepared as described in Fig. 3 legend, from crystalline pancreatic glucagon provided by Novo Laboratories (Paris, Copenhagen). Ca^{2+} uptake was assayed in inside-out plasma membrane vesicles as previously described[1]. Preincubation at 37 °C for 5 min of plasma membrane vesicles (120-320 μg protein) with glucagon(19-29) (0-50 nM) diluted in 50 mM Tris-HCl, *p*H 8, 0.01% bovine serum albumin (BSA) and 0.87 μM lactose, allowed the peptide to interact with intravesicular sites of inside-out vesicles because of the leakiness of vesicles. After 5 min, the incubation was run in 200 μl of assay medium containing 50 mM Tris HCl, *p*H 8, 0.01% BSA, 400 μM EGTA, 393 μM $CaCl_2$ (0.1 μM free Ca^{2+}), 2 μCi ml^{-1} of ^{45}Ca, 180 mM sucrose, 1 μM ruthenium red, 20 mM sodium azide, 4 mM Tris-oxalate, 10 mM $MgCl_2$, with either no ATP added or 10 mM ATP. $MgCl_2$ in the assay medium is used to seal vesicles[1,28]. After 15 min, 150 μl samples were filtered under vacuum on Millipore filters (HAWP 0.45 μM) that had been soaked in 0.25 M KCl for 1 h. The filters were washed three times with 5 ml iced 250 mM sucrose, 40 mM NaCl, dried, and counted in 10 ml of Beckman Ready Solv. The ATP-dependent Ca^{2+} uptake was determined from the difference in radioactivity bound to the filter in the presence and absence of ATP. Uptake is expressed as a percentage of control ATP-dependent Ca^{2+} uptake (6.1 ± 1.7 nmol Ca^{2+} per mg protein per 15 min).

The ATP-dependent Ca^{2+} uptake was studied in liver plasma membrane vesicles prepared according to Prpic *et al.*[7]. Addition of glucagon(19-29) (0-50 nM) to the assay medium resulted in a dose-dependent inhibition of the ATP-dependent Ca^{2+} uptake (Fig. 1). A maximal inhibition of 40-60% was attained with 10 nM glucagon(19-29), half-maximal inhibition occurring at 3 ± 0.3 nM glucagon(19-29).

From kinetic studies[1] and reconstitution experiments using the purified enzyme (in preparation), we have concluded that the high-affinity (Ca^{2+}-Mg^{2+}) ATPase is the enzyme responsible for Ca^{2+} transport. Accordingly, (Ca^{2+}-Mg^{2+}) ATPase activity was assayed in purified liver plasma membranes prepared according to Neville[8] up to step 11. This preparation was chosen because it has been used for the characterization of numerous membrane enzymes and receptors[9-13]. Glucagon(19-29) induced a dose-dependent inhibition of the (Ca^{2+}-Mg^{2+}) ATPase activity (Fig. 2). Maximal inhibition was 15-20% at 10 nM glucagon(19-29), and half-maximal inhibition occurred at 0.75 ± 0.25 nM glucagon(19-29). Inhibition was due to a decrease in maximal velocity of the reaction with no change in the apparent affinity of the enzyme for calcium (data not shown). Also, (Ca^{2+}-Mg^{2+}) ATPase was assayed in parallel with Ca^{2+} uptake in liver plasma membrane vesicles prepared according to Prpic *et al.*[7]. In these preparations, inhibition of (Ca^{2+}-Mg^{2+}) ATPase by glucagon(19-29) reached 15-20% at 10 nM glucagon(19-29), half-maximal inhibition being observed for 1 ± 0.25 nM glucagon(19-29) (data not shown).

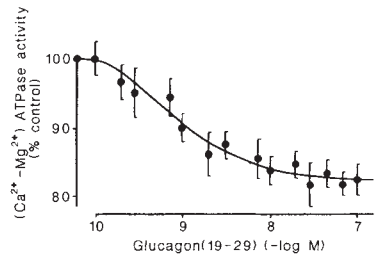

Fig. 2 Inhibition of (Ca^{2+}-Mg^{2+}) ATPase activity in purified liver plasma membranes by glucagon(19-29). Results are the mean of ten different experiments done with five different membrane preparations; bars, ±s.e.m.

Methods. Purified plasma membranes were prepared from liver of female albino Wistar rats (100-120 g body weight) according to Neville[8] up to step 11. Plasma membranes (4-8 μg protein) were preincubated at 4 °C with glucagon(19-29) (0-0.1 μM) diluted in 50 mM Tris-HCl, *p*H 8, 0.01% BSA and 0.87 μM lactose. After 10 min, (Ca^{2+}-Mg^{2+}) ATPase activity was assayed as previously described[1]. The assay medium contained in a final volume of 200 μl, 0.25 mM ATP, 50 mM Tris-HCl, *p*H 8, 400 μM EGTA and 393 μM $CaCl_2$ (0.1 μM free Ca^{2+}). After 10 min incubation at 30 °C, aliquots were assayed for P_i by colorimetric determination[29]. Basal activity was estimated for each point by omitting $CaCl_2$ from the assay medium and (Ca^{2+}-Mg^{2+}) ATPase activity, calculated by subtracting values obtained in the presence of chelator alone from those obtained with chelator plus $CaCl_2$, is expressed as a percentage of control (Ca^{2+}-Mg^{2+}) ATPase activity (1.2 ± 0.2 nmol P_i per mg protein per 10 min).

Native glucagon caused 25 and 50% maximal inhibition of liver (Ca^{2+}-Mg^{2+}) ATPase and Ca^{2+} transport respectively, but its apparent affinity for the Ca^{2+} pump is in the micromolar range (ref. 1, Table 1). Therefore, it appears that glucagon(19-29) is 1,000-fold more efficient than native glucagon in inhibiting the liver Ca^{2+} pump. To assess the specificity of the action of glucagon(19-29), we investigated the action of three other glucagon fragments: glucagon(18-29), glucagon(22-29) and glucagon(1-21). Glucagon(18-29) was tenfold less potent than glucagon(19-29) in inhibiting (Ca^{2+}-Mg^{2+})ATPase (Table 1 and Fig. 3). Glucagon(22-29) produced only a 5-15% maximal inhibition of (Ca^{2+}-Mg^{2+}) ATPase, with a low potency (1 μM), similar to that of native glucagon (Table 1). The amino-terminal (1-21) fragment of glucagon had no effect on (Ca^{2+}-Mg^{2+}) ATPase (Table 1) nor did it antagonize inhibition induced by either native glucagon or glucagon(19-29).

Table 1 Effects of various glucagon fragments on (Ca^{2+}-Mg^{2+}) ATPase activity in liver plasma membranes

	Glucagon(19-29)	Glucagon(18-29)	Glucagon	Glucagon(22-29)	Glucagon(1-21)
K_i (nM)	0.75 ± 0.5	7.5 ± 2.5	700 ± 300	1,000 ± 300	10,000
Inhibition (% maximal)	20 ± 5	15 ± 5	25 ± 10	10 ± 5	—

The (Ca^{2+}-Mg^{2+}) ATPase activity was measured as in Fig. 2 legend, in the presence of 0-10 μM of either glucagon(19-29), glucagon(18-29), glucagon, glucagon(22-29) or glucagon(1-21). A 10-min preincubation at 4 °C of plasma membranes (3-8 μg protein) with the peptide was used, followed by a 10-min incubation at 30 °C, under the conditions described in the legend to Fig. 2. Results are the mean of four different experiments using three different plasma membrane preparations, and are expressed as a percentage of control activity (1.3 ± 0.2 μmol Pi per mg protein per 10 min). Sources: glucagon(1-21), a gift from Novo Laboratories; glucagon(22-29) from Peninsula Laboratories; glucagon(19-29) and glucagon(18-29), prepared from glucagon as described in Fig. 3 legend.

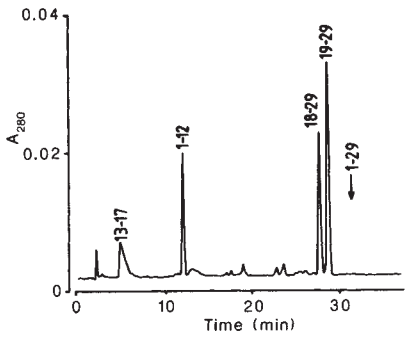

Fig. 3 Preparation of glucagon(19-29) and glucagon(18-29) by HPLC. Arrow, elution volume of intact glucagon(1-29).
Methods. Crystalline porcine pancreatic glucagon was digested for 1 h at 37 °C in 50 mM Tris HCl, 1 mM $CaCl_2$, *p*H 7.5, with tosyl-phenylethyl-chloromethyl-ketone (TPCK)-treated trypsin (Sigma) at a 1:100 (w/w) enzyme/substrate ratio. After boiling for 3 min, the peptide mixture was separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters apparatus equipped with a μBondapak C_{18} column run at 1.5 ml min^{-1} in 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient (0-50% in 50 min) of acetonitrile containing 0.05% ethylacetate[30]. Peptides were monitored by UV absorption at 214 and 280 nm, UV spectra were obtained on-line from the HPLC peaks using a Hewlett-Packard model 8450A diode array spectrophotometer. The purity of the peptide fragments was assessed by comparing their UV spectra (not shown) with theoretical spectra obtained with a computer databank (D.B. and M.D., manuscript in preparation) and by amino-acid analysis performed with RP-HPLC of OPA derivatives[31]. The results obtained (theoretical values in parentheses) were: glucagon(19-29): Ala 1.1 (1), Asp 2.2 (2), Glu 2.4 (2), Phe 1.3 (1), Leu 1.2 (1), Met 0.8 (1), Arg 0.14 (0), Thr 1.1 (1), Val 0.9 (1); Glucagon(18-29): Ala 1.3 (1), Asp 2 (2), Glu 2.2 (2), Phe 1.1 (1), Leu 0.9 (1), Met 0.8 (1), Arg 1.3 (1), Thr 1.3 (1), Val 0.9 (1). The presence of intact tryptophan in both peptides was assessed by their UV spectra (not shown).

The integral glucagon molecule is required for full activation of adenylate cyclase. In particular, neither glucagon(1-21)[14] nor glucagon(22-29)[15] can stimulate adenylate cyclase activity. We did not observe any effect of glucagon(19-29) (0-0.1 mM) on adenylate cyclase activity (not shown). This is in keeping with our previous observation that inhibition of the Ca^{2+} pump by glucagon is independent of cyclic AMP production[1].

Our results clearly indicate that nanomolar concentrations of glucagon(19-29) specifically inhibit the Ca^{2+} pump in liver plasma membranes. Furthermore, the present data show that the amino-terminal residues in glucagon(19-29) are essential. Indeed, the addition of an arginine residue (peptide (18-29)) or the suppression of residues 19-21 (peptide (22-29)) resulted in a dramatic loss of potency. Thus, glucagon(19-29) appears to be the molecular form of glucagon responsible for the inhibition of the calcium pump in the liver plasma membrane. The metabolic consequences of the action of glucagon(19-29) on liver cells, in particular its effect on the glycogenolytic pathway, remain to be elucidated, as does the nature of the specific receptor which recognizes this peptide.

Two hypotheses can be raised concerning the physiological precursor of glucagon(19-29). Firstly, glucagon(19-29) could be released during the processing of proglucagon as are other glucagon-related peptides[16-20]. Alternatively, glucagon(19-29) could be generated locally upon interaction of the circulating glucagon with hepatocytes. The existence of a trypsin-like protease associated with liver plasma membranes has been demonstrated[21]. Conversion of peptide hormones by the target tissue has already been described for growth hormone[22], proinsulin[23], angiotensin 1[24], and arginine vasopressin[25]. Also, cleavage of growth hormone[26] and arginine vasopressin[25] has been shown to generate new biological activities.

It was generally accepted that adenylate cyclase activation was the only enzymatic pathway regulated by glucagon. Wakelam *et al.*[27] recently reported that subnanomolar concentrations of glucagon cause a breakdown of inositol phospholipids independently of cyclic AMP production suggesting that glucagon may also regulate phospholipase C. Our findings provide a new mechanism by which glucagon may regulate liver cell metabolism through an enzymatic pathway distinct from adenylate cyclase or phospholipase C, and point out the possible role of glucagon as a precursor of peptides with new biological activities.

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Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence

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Eukaryotic messenger RNAs are translated with unequal efficiencies *in vivo*[1,2] and *in vitro*[3-5] and the molecular basis of this phenomenon is not understood. As an approach to understanding the role of the 5′ untranslated leader sequence in regulating mRNA translational efficiency, chimaeric mRNAs have been generated by joining a heterologous leader to complementary DNA (cDNA) sequences, followed by *in vitro* transcription using SP6 RNA polymerase and *in vitro* protein synthesis. We used the untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV