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THE ROLE OF THE AUTONOMIC INNERVATION IN THE CONTROL OF GLUCAGON RELEASE DURING HYPOGLYCAEMIA IN THE CALF

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SUMMARY

1. The extent to which the autonomic innervation to the pancreas is implicated in the control of glucagon release during hypoglycaemia has been investigated in calves 3–6 weeks after birth.

2. A pronounced rise in plasma glucagon concentration occurred in normal conscious calves in response to hypoglycaemia following administration of insulin (0·1 u./kg). Prior treatment with atropine caused no significant change in the hypoglycaemic response to insulin in these animals but the rise in plasma glucagon concentration was delayed.

3. Section of both splanchnic nerves produced no significant change in the tolerance of conscious calves to this small dose of insulin and the changes in plasma glucagon concentration in these animals were within the normal range.

4. In contrast, the same dose of insulin produced severe hypoglycaemia, accompanied by convulsions, in atropinized calves with cut splanchnic nerves. In spite of the intensity of the hypoglycaemic stimulus the rise in plasma glucagon concentration was both delayed and diminished in these animals.

5. Administration of atropine alone (0·2 mg/kg) to normal fasting calves produced a significant fall in the mean plasma concentrations of both glucose and glucagon ($P < 0·01$) within 30 min, without affecting that of insulin.

6. A significant increase in plasma glucagon concentration also occurred in response to stimulation of the peripheral ends of the thoracic vagi in adrenalectomized calves with cut splanchnic nerves under barbiturate anaesthesia. A rise in mean plasma glucose concentration was also observed in these experiments and found to be significantly correlated with the glucagon response.

7. It is concluded that changes in either sympathetic or parasympathetic efferent activity may modify plasma glucagon concentration in the conscious calf, but that only the latter mechanism is likely to be implicated in the response to changes in plasma glucose concentration within the physiological range.

INTRODUCTION

It has long been known that the pancreatic islets are richly innervated by autonomic fibres (Langerhans, 1869; Van Campenhout, 1927; Munger, 1972) and several workers have obtained evidence that glucagon is released from the A cells in response to adrenergic stimulation (Esterhuizen & Howell, 1970; Leclercq-Meyer, Brisson & Malaisse, 1971; Marliss, Girardier, Seydaux, Kanazawa, Wollheim, Orei & Porte, 1972). Stimulation of the sympathetic innervation to the pancreas in anaesthetized calves causes a significant rise in peripheral plasma glucagon concentration at frequencies as low as 0·5 c/s (Bloom, Edwards & Vaughan, 1973) and the effect is approximately linearly related to stimulus frequency over the range 0–10 c/s. These results suggest that tonic variations in sympathetic efferent activity are likely to influence the rate of release of this hormone but provide no information about the sensitivity of the sympathetic system to variations in plasma glucose concentration within the normal physiological range.

The experiments which are described in the present paper were undertaken in order to ascertain the extent to which the autonomic nervous system is involved in the release of glucagon which occurs during moderate hypoglycaemia in the conscious calf. The results show that, whereas the sympathetic system is relatively insensitive to this specific stimulus, a cholinergic mechanism is functionally important in this respect under these conditions.

Certain of these results have been published previously in preliminary form (Vaughan, Bloom, Ogawa, Bircham & Edwards, 1973).

METHODS

Animals

The experiments were carried out on pedigree Jersey calves aged 20–40 days and maintained on a milk diet (6–8 pints/day); food was withheld for at least 14 hr before each experiment.

Experimental procedures

Conscious calves were kept unrestrained in individual pens. Blood samples were withdrawn at intervals from a braunula cannula previously inserted into the jugular vein under local anaesthesia (2 % Xylocaine; Astra Chemicals Ltd). Samples destined for glucagon or insulin assay were treated with aprotonin (Trasylol: F.B.A. Labora-

tories, 10,000 K.I.U./ml.) before centrifugation (10% dilution, v/v) and the plasma was stored at −20° C.

Insulin tolerance tests. Insulin for I.V. injection was prepared by dissolving 12 mg of 'six times recrystallized' bovine insulin (Boots Pure Drug Co. Ltd) (biological potency; 24·4 i.u./mg) in 5–10 ml. 0·01 N-HCl and then adding 0·9% NaCl (w/v) to provide a final dilution of 12 mg/100 ml. Animals were observed continuously throughout each experiment. When required, section of both splanchnic nerves was carried out under general anaesthesia at least 3 days before animals were tested with insulin. Normal aseptic precautions were observed and the animals were given penicillin daily between operation and experiment. Only one experiment was carried out on any individual calf.

In some experiments atropine (atropine sulphate; B.D.H.) was given to normal fasting calves by I.V. injection (0·9% (w/v) NaCl, 0·1 g/100 ml.) at a dose of 0·2 mg/kg, 30 min before administration of insulin.

Vagal stimulation. Animals were anaesthetized with sodium pentobarbitone (May & Baker) administered by intravenous injection (0·9% (w/v) NaCl, 6 g/100 ml.). Both adrenal glands were then removed, the splanchic nerves were divided at the level of the diaphragm and the pylorus was ligated. Lateral thoracotomy was performed after which respiration was maintained by intermittent positive pressure ventilation. Both dorsal and ventral vagi were identified below the heart and the peripheral ends were attached to separate fluid electrodes. In each experiment the nerves were stimulated at either 5·0 or 20·0 c/s for 10 min. Blood pressure was measured continuously by means of a pressure transducer connected to a polyethylene catheter which was inserted into the femoral artery so that the tip lay in the abdominal aorta. Blood samples were withdrawn from this catheter for analysis as required.

Estimations

Plasma glucose was estimated with glucose oxidase, either by means of the Beckman Glucose Analyzer, or according to Huggett & Nixon (1957); lactic acid was estimated enzymically as described by Barker & Britton (1957). Plasma glucagon was measured by radioimmunoassay using a highly specific antibody which showed no cross-reaction with enteroglucagon. Insulin was measured as previously described (Bloom, Edwards & Vaughan, 1973). At the conclusion of experiments in anaesthetized animals small pieces of liver were removed for glycogen analysis as described previously (Edwards, 1971). Animals in which the liver glycogen concentration was below 5 mg/g at the end of the experiment were omitted from the series.

Statistical analyses were made according to the methods of Snedecor & Cochran (1967).

RESULTS

Insulin tolerance tests

Intravenous injections of bovine insulin (0·1 u./kg) caused an abrupt fall in plasma glucose concentration in normal conscious calves. Thus the mean concentration had fallen by $72 \pm 3\%$, from a resting value of 86 ± 5 mg/100 ml. ($n = 4$), within 30 min; thereafter mean plasma glucose rose steadily to within 10% of the initial value at 150 min. Hypoglycaemia was accompanied by a prompt rise in mean plasma glucagon concentration which rose from a resting value of 124 ± 44 pg/ml. by a mean maximum increment of 219 ± 43 pg/ml. at 45 min. The animals were already re-

covering from hypoglycaemia at this time and the mean plasma glucagon concentration fell steadily towards the resting value during the remainder of the experiment (Fig. 1).

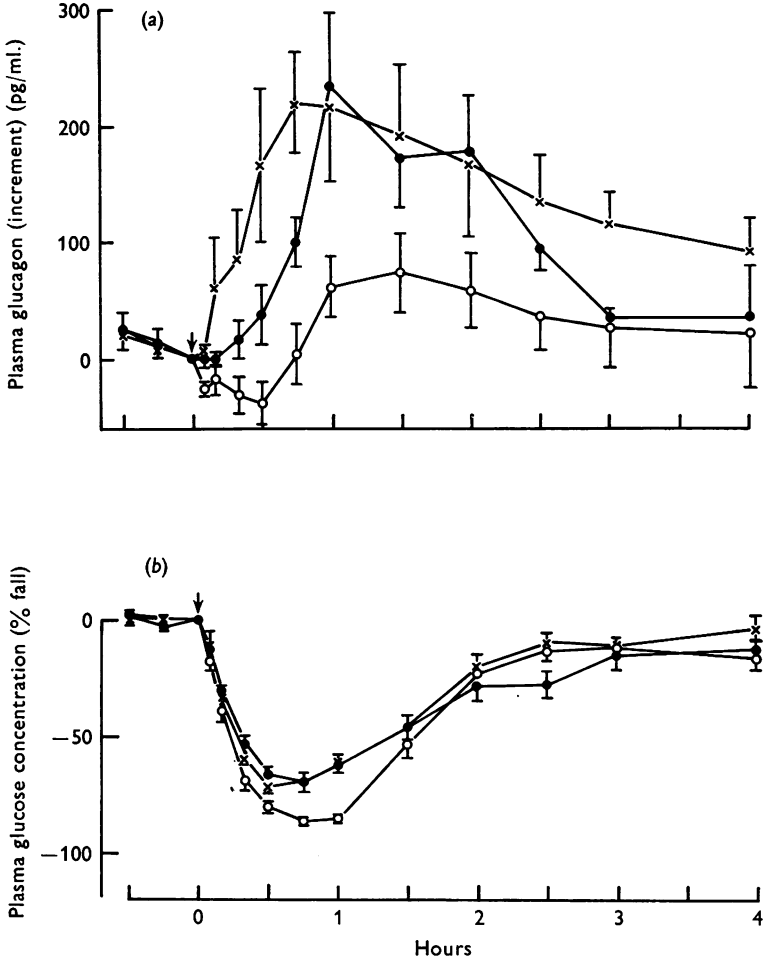

Fig. 1. Comparison of the changes in the concentrations of (*a*) glucagon and (*b*) glucose in the plasma of conscious calves in response to insulin (0·1 u./kg). ×, Normal control animals ($n = 4$). ●, splanchnic nerves cut ($n = 5$); ○, splanchnic nerves cut, pre-treated with atropine ($n = 7$). Vertical bars: S.E. of each mean value. Insulin was injected at the arrow.

The tolerance to insulin was not significantly altered by previous section of both splanchnic nerves. Mean plasma glucose concentration had fallen by 64 ± 6 % at 30 min and plasma glucagon concentration had risen by a mean maximum increment of 234 ± 63 pg/ml. at 60 min (Fig. 1). The

mean absolute values at time = 0 did not differ significantly from those of the control group (glucose: 90 ± 7 mg/100 ml.; glucagon: 193 ± 56 pg/ml.; $n = 5$). Whereas the rise in plasma glucagon concentration was found to be slightly delayed by comparison with the control group, the response did not differ significantly from normal ($P > 0{\cdot}05$).

In contrast, administration of atropine to calves with cut splanchnic nerves substantially reduced their tolerance to insulin and significantly

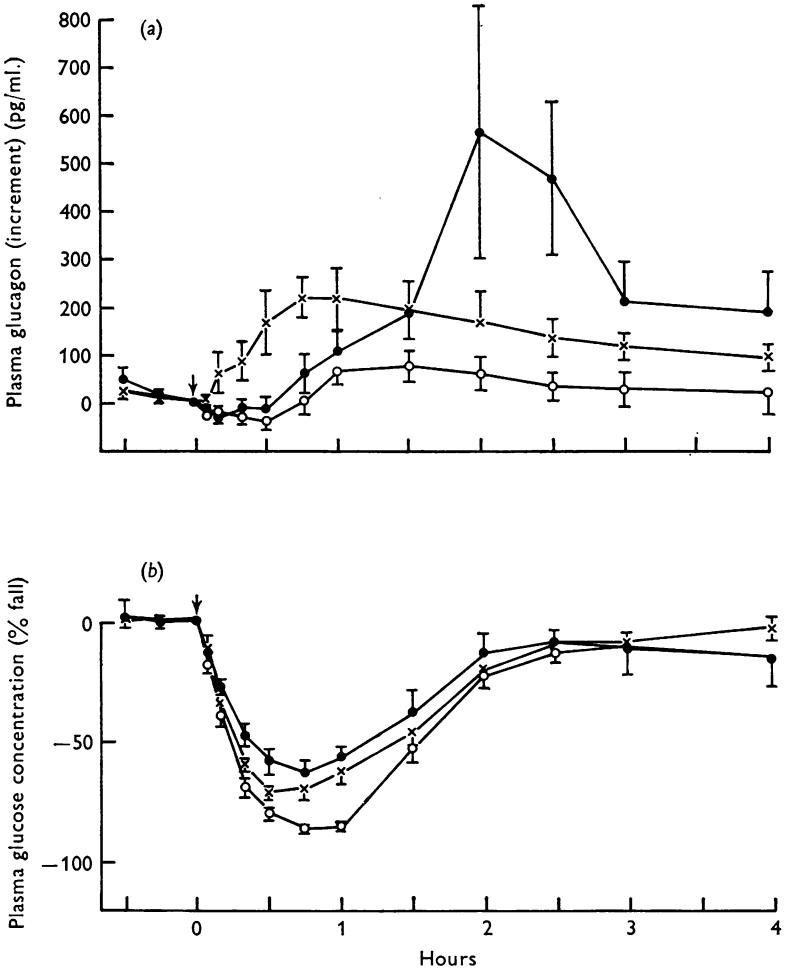

Fig. 2. Comparison of the changes in the concentrations of (*a*) glucagon and (*b*) glucose in the plasma of conscious calves in response to insulin (0·1 u./kg). ×, Normal control animals ($n = 4$); ●, splanchnic nerves intact, pre-treated with atropine ($n = 5$); ○, splanchnic nerves cut, pre-treated with atropine ($n = 7$). Vertical bars: S.E. of each mean value. Insulin was injected at the arrow.

diminished the rise in plasma glucagon concentration. Hypoglycaemia was intensified and prolonged in these animals; mean plasma glucose concentration had fallen by $85 \pm 2\%$ at 60 min from a mean resting value of 89 ± 5 mg/100 ml. ($n = 7$). This fall was significantly greater than that

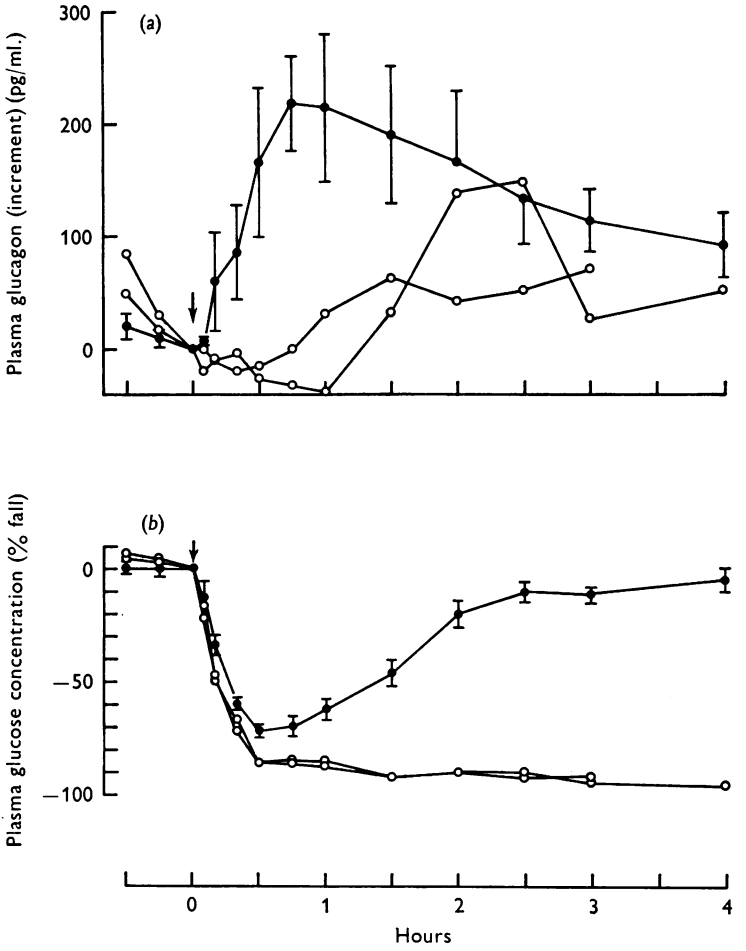

Fig. 3. Comparison of the changes in the concentrations of (*a*) glucagon and (*b*) glucose in the plasma of conscious calves in response to insulin. ●, Normal control animals given 0·1 u./kg ($n = 4$); ○, two animals with cut splanchnic nerves, pre-treated with atropine and given 4 u./kg. Vertical bars: S.E. of each mean value. Insulin was injected at the arrow.

in either of the other two groups ($P < 0{\cdot}001$). Furthermore, each of these animals convulsed 30–60 min after receiving insulin whereas neither convulsions nor any other visible signs of hypoglycaemia were observed in

other animals given this relatively small dose of insulin. In spite of the severity of the hypoglycaemia which occurred under these conditions the rise in mean plasma glucagon concentration was both delayed and diminished. At 90 min mean plasma glucagon concentration had risen by only 74 ± 33 pg/ml. from an initial value of 141 ± 26 pg/ml. (Fig. 1). Each of the incremental values during the first 60 min were significantly lower than those in the normal control animals ($P < 0{\cdot}01$) and, with the single exception of the 10 min value, significantly lower than those for the group with cut splanchnic nerves which did not receive atropine ($P < 0{\cdot}01$).

Administration of the same dose of atropine (0·2 mg/kg) to normal calves with intact splanchnic nerves produced no significant change in the hypoglycaemic response to insulin (Fig. 2). However, the rise in mean plasma glucagon concentration which is normally associated with the initial stages of hypoglycaemia was abolished and the incremental values during the first 60 min after insulin did not differ significantly from those in atropinized calves with cut splanchnic nerves (Fig. 2). Thereafter the atropinized normal calves showed a delayed mean rise in plasma glucagon concentration of considerable magnitude, but there were wide variations between individuals. This delayed and variable response was abolished by prior section of both splanchnic nerves.

The possibility that glucagon might still be released after complete autonomic blockade, if the hypoglycaemic stimulus were sufficiently intense, was examined in two atropinized calves with cut splanchnic nerves which were given a much larger dose of insulin (4 u./kg).

Plasma glucose concentration had fallen below 10 mg/100 ml. within 30 min in both animals and showed no tendency to rise thereafter. Both calves convulsed 30–40 min after insulin was injected; one died at 210 min and the plasma lactate concentration of the other had risen from 18·2 to more than 150 mg/100 ml. by 240 min. In spite of the intensity of the hypoglycaemic stimulus achieved in these experiments the rise in plasma glucagon concentration which occurred during the first 45 min after administration of 0·1 u. insulin to normal control animals was abolished (Fig. 3).

Effect of atropine alone in fasting calves

The changes in plasma glucose, insulin and glucagon concentration which occurred in response to a single intravenous injection of atropine (0·2 mg/kg) were examined in fourteen normal conscious calves (Fig. 4).

The concentrations of glucagon and glucose in the venous plasma both fell steadily during the 30 min after atropine was injected, whereas that of insulin was apparently unaffected. Thus, mean plasma glucose concentration had fallen by $6{\cdot}9 \pm 1{\cdot}6$ mg/100 ml. from an initial value of

82 ± 3 mg/100 ml. during this period ($P < 0{\cdot}01$) and mean plasma glucagon concentration by 42 ± 10 pg/ml. from a value of 140 ± 22 pg/ml. at time = 0 ($P < 0{\cdot}01$).

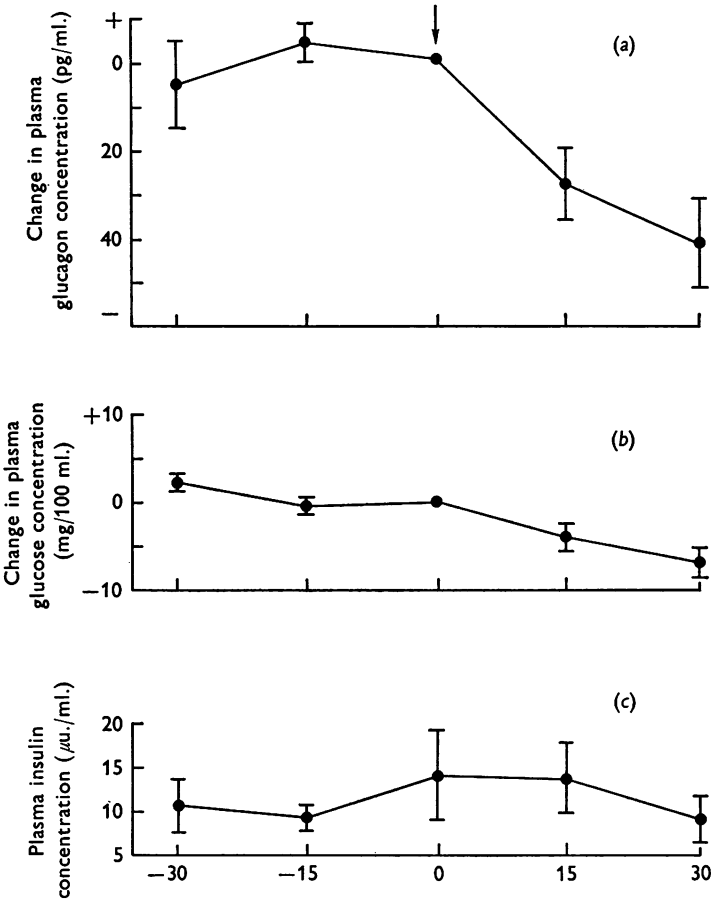

Fig. 4. Changes in the mean concentrations of (*a*) glucagon, (*b*) glucose and (*c*) insulin in the venous plasma of fourteen conscious calves given atropine I.V. (0·2 mg/kg). Vertical bars: S.E. of each mean value. Atropine was injected at the arrow.

Effects of vagal stimulation

Stimulation of the peripheral ends of the thoracic vagi in adrenalectomized calves, with cut splanchnic nerves, caused an abrupt rise in mean plasma glucagon concentration. Stimulation at 5·0 c/s produced a rise of 42 ± 7 pg/ml. ($n = 4$) and at 20 c/s of 57 ± 16 pg/ml. ($n = 5$) at 10 min, when stimulation was discontinued. Plasma glucagon fell steadily towards the resting level during the next 10 min but tended to drift higher

thereafter and substantial individual variation was encountered (Fig. 5*a*). The rise in mean plasma glucagon concentration during nerve stimulation represented a significant increase above the resting values in both groups ($P < 0{\cdot}05$) and amounted to an increase of 50–60% above the initial concentration. Mean plasma glucose concentration also rose steadily during stimulation and fell toward the resting value during the next 10 min along a very similar time course to that for glucagon (Fig. 5*b*). These changes in mean plasma glucose and glucagon concentration were found to be highly correlated (5 c/s: $r = 0{\cdot}79$; 20·0 c/s: $r = 0{\cdot}90$) during the period 0–20 min.

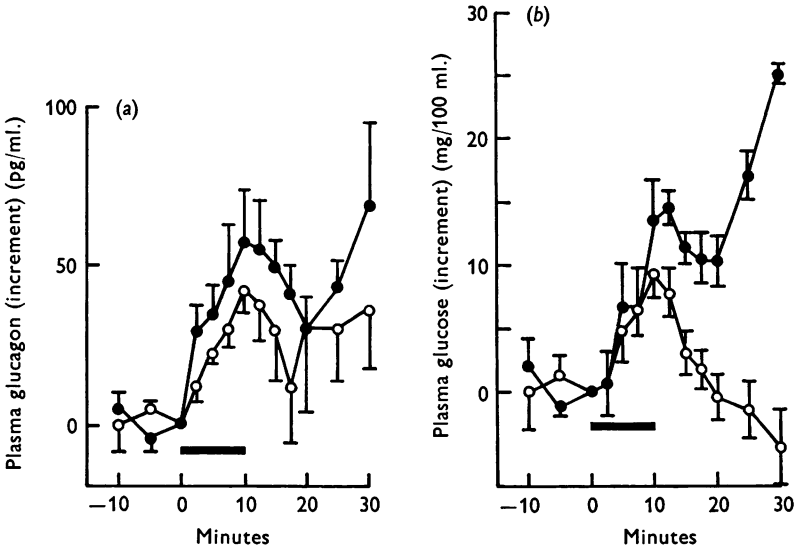

Fig. 5. Comparison of the changes in the concentrations of (*a*) glucagon and (*b*) glucose in the arterial plasma of adrenalectomized calves, with cut splanchnic nerves, in response to stimulation of the peripheral ends of both vagi in the thorax. ○, 5·0 c/s ($n = 4$); ●, 20·0 c/s ($n = 5$). Vertical bars: S.E. of each mean value. Horizontal bars: duration of stimulus.

The interpretation of these results is complicated by the systemic effects of the preparatory surgical procedures. Bilateral adrenalectomy, section of both splanchnic nerves, together with the necessity of maintaining respiration by positive pressure ventilation combined to produce a low aortic pressure (70–80 mmHg) at the start of the experiments. stimulation of both vagus nerves invariably produced a further substantial fall in blood pressure and circulatory failure supervened in several animals after 40–50 min. It therefore seems likely that the delayed rises in plasma glucagon and glucose concentration, which occurred in many of these experiments, were due to non-specific effects consequent upon tissue hypoxia.

DISCUSSION

Previous studies have shown that the hepatic glycogenolytic mechanism is extremely sensitive to stimulation via the sympathetic system in the calf 3–6 weeks after birth. At least three separate mechanisms are involved in this response; sympathetic efferent fibres which supply the liver directly (Edwards & Silver, 1970), catecholamines released from the adrenal medullae (Edwards & Silver, 1972) and glucagon released from the pancreas in response to stimulation of the splanchnic sympathetic innervation (Bloom, Edwards & Vaughan, 1973). The young calf would therefore appear to be the species of choice in which to assess the extent to which the sympathetic system is involved in the homoeostatic responses to changes in plasma glucose concentration within the normal physiological range.

The results of the present experiments show that the sympathetic system is relatively insensitive to hypoglycaemia in the calf. Both the extent and duration of the fall in plasma glucose concentration which occurred in response to a small dose of insulin, in calves in which both splanchnic nerves had previously been cut, were closely similar to those in normal calves of the same age. Furthermore the changes in mean plasma glucagon concentration did not differ significantly in these two groups of animals. In three of the five normal calves pre-treated with atropine, the same dose of insulin (0·1 u./kg) produced a very substantial increase in plasma glucagon concentration (increments ranging between 370 and 1460 pg/ml.) after a delay of between 90 and 120 min. This delayed and variable rise in plasma glucagon concentration was not observed in animals given this dose of insulin after section of both splanchnic nerves. It therefore seems likely that the hypoglycaemia which results from the administration of 0·1 u insulin/kg is close to the threshold stimulus necessary to activate release of pancreatic glucagon via the sympathetic innervation in atropinized calves.

The finding that stimulation of the peripheral ends of the vagus nerves produces a rise in plasma glucagon concentration is of considerable interest in view of the close proximity of both adrenergic and cholinergic nerve terminals to the alpha cells in the pancreatic islets (Renold, 1971). It would appear that the rise in plasma glucose concentration during these experiments was due to the release of glucagon since the changes in concentration of both were found to be closely correlated. Furthermore, comparable changes in plasma glucagon concentration, induced by intraportal infusions of exogenous glucagon, produce hyperglycaemia of approximately the same magnitude in anaesthetized calves (Bloom, Edwards & Vaughan, 1973). However, these results are susceptible to criticism, not only by

virtue of the limited viability of the preparation, but also because of the widespread nature of the stimulus and the consequential opportunities for initiation of secondary and indirect effects. Accordingly, this observation provides no more than a tentative suggestion for the existence of a cholinergic mechanism which may be implicated in the control of glucagon release from the pancreas. It is unfortunate that the dependence of the complex ruminant stomach upon tonic vagal activity, to maintain normal function, precludes an effective analysis of the responses of conscious vagotomized calves. However, recent work in human subjects shows that vagotomy significantly reduces both the fasting plasma glucagon concentration together with the rise in that concentration which normally occurs in response to hypoglycaemia (S. R. Bloom, C. Russell & N. J. A. Vaughan, unpublished observations).

The results obtained in conscious calves, pretreated with atropine, provide much stronger evidence in favour of this contention and indicate that the cholinergic mechanism is more sensitive to hypoglycaemia than the sympathetic system. Administration of atropine was found to abolish the rise in plasma glucagon concentration which occurs in response to low plasma glucose concentration in calves with cut splanchnic nerves, however intense the hypoglycaemic stimulus. Evidence was also obtained to show that the glucagon which is normally released in response to moderate hypoglycaemia, in the absence of atropine, participates in restoring the plasma glucose concentration. Thus both the duration and intensity of insulin hypoglycaemia was increased by atropine in calves with cut splanchnic nerves and convulsions invariably occurred under these conditions. The fall in the concentration of both glucagon and glucose in the peripheral plasma, which occurred in normal conscious calves in response to atropine, suggests that this pathway is also implicated in the regulation of glucagon release from the pancreas under resting conditions.

The results obtained in calves given a large dose of insulin are not directly comparable with those from experiments designed to elucidate the response to moderate hypoglycaemia as the very high insulin concentrations may have caused a direct suppression of the alpha cells. However, it is worth noting that the prolonged and severe hypoglycaemia which occurred in these atropinized calves with cut splanchnic nerves after administration of 4 u. insulin/kg did produce a comparatively small change in plasma glucagon concentration. The results of the experiments *in vivo* accord with *in vitro* studies, since isolated pancreatic islets release glucagon only in response to extreme fluctuations in the concentration of glucose in the surrounding medium (Edwards, Howell & Taylor, 1969; Iversen, 1971) although the addition of either adrenaline or acetylcholine to these pre-

parations causes a very large increase in glucagon output (Leclercq-Meyer *et al.* 1971; Iversen, 1971).

It would appear that the glucoregulatory centres in the brain (Bernard, 1849) stimulate release of pancreatic glucagon via the autonomic innervation to the islets. Both divisions of the autonomic nervous system may influence the rate at which the hormone is released under normal conditions but, whereas the alpha cells respond readily to this form of stimulation, they are comparatively resistant to changes in the concentration of glucose in the surrounding extracellular fluid. These results in calves show that the release of glucagon in response to the specific stimulus provided by moderate hypoglycaemia is mediated via the parasympathetic innervation to the pancreas. Previous studies indicate that any non-specific stimulus, of sufficient intensity to impose 'stress' or cause a generalized increase in sympathetic efferent activity will also stimulate the release of this hormone from the pancreatic islets (Bloom, Daniel, Johnston, Ogawa & Pratt, 1973; Bloom, Edwards & Vaughan, 1973). Such a conclusion is in agreement with generally accepted views of the different roles that the two divisions of the autonomic nervous system fulfil.

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