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# Expression of immediate early genes in rat gastric myenteric neurones: a physiological response to feeding

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1. Expression of the immediate early genes c-*fos*, c-*jun* and c-*myc* in rat stomach in response to feeding and gastric distension was examined by Northern blot analysis and *in situ* hybridization.

2. Refeeding of fasted rats induced a transient increase in c-*fos* mRNA abundance in gastric corpus and antrum that was sixfold within 15 min and declined within 4 h. The response was not mediated by gastrinergic or muscarinic cholinergic mechanisms; it was reduced but not abolished by hexamethonium. No changes in expression of c-*jun*, c-*myc* or the constitutively expressed protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were observed.

3. In conscious rats prepared with a gastric fistula, gastric distension with nutritive and non-nutritive solutions at a physiological pressure for 30 min induced expression of c-*fos*, c-*jun* and c-*myc*, but not GAPDH.

4. Messenger RNA encoding c-*fos* was localized by *in situ* hybridization to gastric myenteric neurones of animals that underwent gastric distension, but not of undistended controls.

5. The results suggest that expression of c-*fos* in gastric myenteric neurones is an early response to the physiological stretching of the stomach wall that accompanies feeding. With supraphysiological distension, other immediate early genes may be recruited.

It is well established that expression of genes encoding regulators of gastrointestinal function is modulated by the contents and pH of the gut lumen over a period of hours to several days. In addition, however, such genes can undergo rapid changes in level of expression with changes in digestive state. For example, gastrin mRNA levels in the antrum of fasted rats are significantly elevated after 1–2 h of refeeding, and somatostatin mRNA is depressed over a similar time course (Wu, Sumii, Tari, Mogard & Walsh, 1990). In the gastric corpus of fasted rats, the abundance of mRNA encoding histidine decarboxylase (HDC), the enzyme that converts histidine to histamine, is doubled within 30 min of refeeding (Dimaline, Sandvik, Evans, Forster & Dockray, 1993). It is not yet clear what factors mediate these feeding-induced changes in gene expression, but their rapidity of onset is reminiscent of immediate early gene expression. It has been shown that refeeding fasted rats a liquid meal induces expression of c-*fos* protein in the brainstem (Fraser & Davison, 1993), in an area associated with responses to gastric distension (Raybould, Gayton & Dockray, 1988). The purpose of the present study was to establish whether gastric expression of immediate early genes was involved in the physiological response to feeding. We examined changes in mRNA encoding c-*fos*, c-*jun* and c-*myc* in rat stomach in response to feeding, and to gastric distension. Cellular localization of c-*fos* was established by *in situ* hybridization. We present evidence that c-*fos* is expressed in gastric myenteric neurones in response to physiological stretching of the stomach wall.

## METHODS

### Animal treatments

Groups of six male rats (200–250 g) were fasted for 48 h (water *ad libitum*). One group received no further treatment, a second group was refed with standard rat pellets for 15 min, and another for 30 min. Four further groups were also refed for 30 min, but 1 h prior to refeeding received either monoclonal antibody (28·2) specific for biologically active gastrin, an antibody (109·21) specific for a non-active gastrin processing intermediate (Yang, Wong, Walsh & Tache, 1989) by tail vein injection under halothane anaesthesia (2·6 mg protein per rat), atropine (600 $\mu$g kg$^{-1}$, s.c.), or hexamethonium (10 mg kg$^{-1}$, s.c.). At the end of the treatment period animals were killed by decapitation, trunk blood collected for gastrin radioimmunoassay (Dockray, Best & Taylor, 1977, 1991) and samples of gastric antrum and corpus removed for total RNA extraction. Three smaller groups of animals ($n = 2$–$3$) were fasted then refed for 2, 4 or 6 h before collection of gastric samples for RNA extraction.

In a separate experiment, sixteen rats were provided with chronic indwelling gastric fistulae under anaesthesia (fluanisone, 5·0; fentanyl, 0·1; midozolam, 2·5 mg $kg^{-1}$, I.P.) as previously described (Dimaline, Carter & Barnes, 1986). Post-operatively, animals received a single injection of Streptopen (0·1 ml, I.M.; Pitman-Moore Ltd). Three weeks after recovery the animals were fasted for 24 h, and the stomachs gently rinsed out. Four rats received no further treatment; in six rats the stomach was distended with 2% methyl cellulose in 0·14 M NaCl for 30 min at a constant pressure of 5 $cmH_2O$; the remaining six animals received 4·5% peptone solution under similar conditions. At the end of the treatment period samples of gastric corpus were taken for RNA extraction, and for *in situ* hybridization. A further group of intact animals was fasted and refed, killed by decapitation and samples of gastric corpus removed for *in situ* hybridization studies.

## Quantification of mRNA

Total RNA was extracted from gastric corpus or antrum using guanidinium isothiocyanate and purified on a caesium chloride cushion as previously described (Dimaline *et al.* 1993). Samples of total RNA (20 μg) were electrophoresed in 1% agarose–formaldehyde gels containing ethidium bromide and electro-transferred to Nylon membranes (Boerhinger-Mannheim). Oligonucleotides for the polymerase chain reaction (PCR) and for *in situ* hybridization were synthesized using an Applied Biosystems 391 PCR-mate (ABS, Warrington, UK). Templates for cRNA synthesis were generated by PCR using oligonucleotides based on the published DNA sequences (Curran, Gordon, Rubino & Sambucetti, 1987; Hayashi, Makino, Kawamura, Arisawa & Yoneda, 1987; Sakai, Okuda, Hatayama, Sato, Nishi & Muramatsu, 1989):

| | |
|---|---|
| c-*fos* sense | 5′-ACACAGGACTTTTGCGCAGAT-3′ |
| c-*fos* antisense | 5′-GAGGTCACAGACATCTCCTCT-3′ |
| c-*jun* sense | 5′-CCTACGGCTACAGTAACC-3′ |
| c-*jun* antisense | 5′-TTGGGGCACAAGAACTGAGTGG-3′ |
| c-*myc* sense | 5′-TCACTGGAACTTACAATCTGCG-3′ |
| c-*myc* antisense | 5′-GAGGTCATAGTTCCTGTTAG-3′ |
| GAPDH sense | 5′-GACCCCTTCATTGACCTCAACT-3′ |
| GAPDH antisense | 5′-CTCAGTGTAGCCCAGGATGCC-3′ |

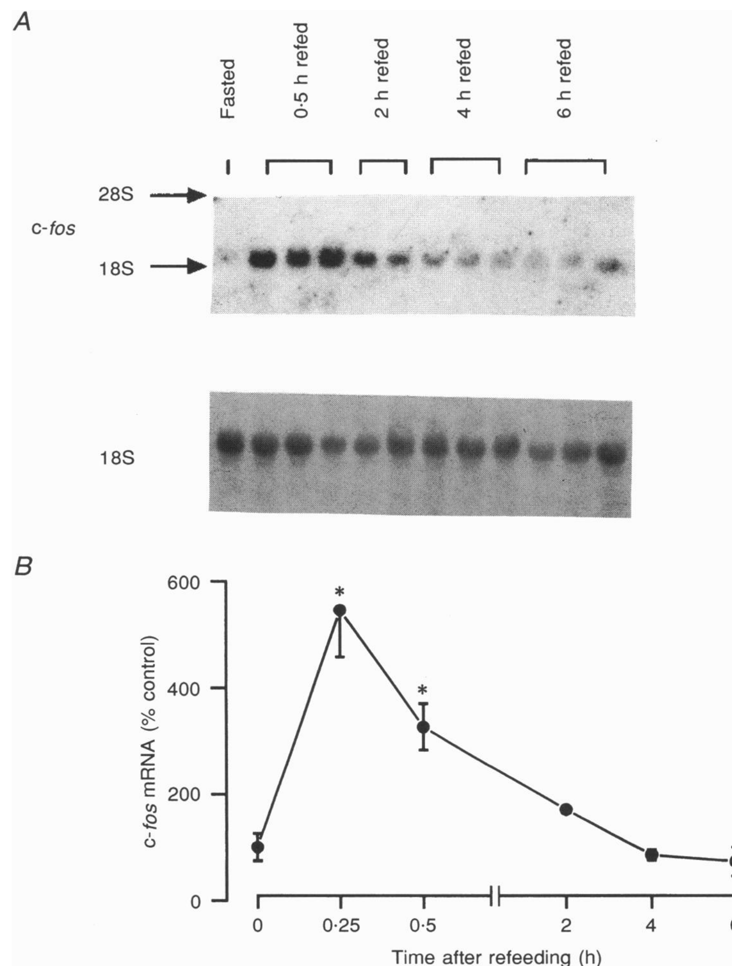


**Figure 1. Time course of c-*fos* expression in response to feeding**

*A*, Northern blot of total RNA from the gastric corpus of rats fasted for 48 h then refed for 0, 0·5, 2, 4 or 6 h. Upper signal, hybridization with c-*fos* cRNA probe; lower signal, hybridization with 18S oligonucleotide probe. *B*, c-*fos* mRNA abundance in fasted rats after feeding for 0 ($n = 6$), 0·25 ($n = 6$), 0·5 ($n = 6$), 2 ($n = 2$), 4 ($n = 3$) or 6 h ($n = 3$). Values are means ± S.E.M.; * $P < 0.05$ relative to time 0, ANOVA.

After subcloning in pCRII (R & D Systems, Abingdon, UK) [$^{32}$P]-labelled cRNA probes were prepared by *in vitro* transcription according to standard protocols. Membranes were first hybridized with the cRNA probe to c-*fos*, washed and exposed to Kodak X-AR film for 48–72 h, or to a storage phosphor screen for up to 24 h and scanned with a PhosphorImager (Molecular Dynamics, Sevenoaks, UK). Probe was removed by boiling in 0·1 % SDS and the membranes sequentially rehybridized with the cRNA probes for c-*jun*, c-*myc* and GAPDH. Membranes were finally hybridized with an end-labelled oligonucleotide probe to the 18S ribosomal subunit to monitor gel loading and transfer efficiency.

### *In situ* hybridization

Samples of gastric corpus were removed from control (48 h fasted) rats, from rats refed following a fast, or from rats subjected to gastric distension with methyl cellulose, quickly frozen flat at −70 °C then placed in isopentane at −40 °C. *In situ* hybridization was performed as previously described (Day, McKnight, Poat & Hughes, 1994). Briefly, sections (10 $\mu$m) were thaw mounted onto polylysyl L-lysine coated slides, fixed with paraformaldehyde, washed and dehydrated. Sections were hybridized with an oligonucleotide (5′-GCAGCGGGAGGATGACGCCTCGTAGTCCG-CGTTGAAACCCGAGAA-3′) end-tailed with $^{35}$S-[dATP], complementary to the region of c-*fos* DNA that encodes the amino-terminal part of the *fos* protein (Curran *et al.* 1987). Hybridizations were performed at 42 °C overnight, the slides washed, dehydrated and dried before exposure to $\beta$-Max film (Amersham); selected sections were dipped with LM1 emulsion (Kodak).

### Data analysis

Northern blots were quantified by scanning densitometry. The abundance of mRNA encoding the immediate early genes is expressed relative to that of the constitutively expressed protein GAPDH. Values are expressed as means ± S.E.M. Statistical significance was evaluated by one-way ANOVA and Student's two-tailed *t* test.

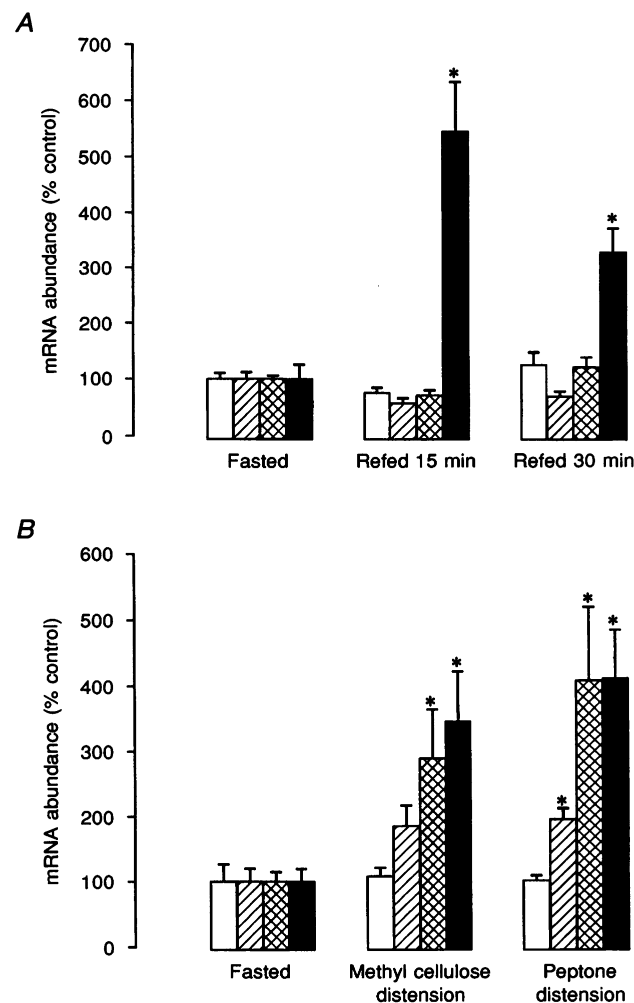


**Figure 2. Time course of c-*fos* expression in response to feeding**

Immediate early gene mRNA abundance in rat gastric corpus. *A*, animals fasted for 48 h or fasted for 48 h then refed for 15 or 30 min. *B*, gastric fistula rats fasted for 48 h or fasted then subjected to gastric distension (5 $cmH_2O$ pressure for 30 min) with 2% methyl cellulose or 4·5% peptone. □, GAPDH; ▨, c-*myc*; ▩, c-*jun*; ■, c-*fos*. * $P < 0·05$ relative to fasted, ANOVA.

## RESULTS

In Northern blots of mRNA from the corpus of rats fed *ad libitum*, the c-*fos* probe revealed a single mRNA species of approximately 2·1 kb whose abundance was reduced threefold by a 48 h fast ($P < 0.005$, Student's *t* test). When fasted animals were refed, the c-*fos* mRNA abundance increased sixfold within 15 min ($P < 0.05$, ANOVA, $n = 6$, Fig. 1). After 30 min c-*fos* mRNA was still elevated threefold ($P < 0.05$) and declined to basal levels by about 4 h (Fig. 1). Similar responses were also seen in gastric antrum (data not shown). Plasma gastrin concentrations were also elevated by refeeding; basal, 14·4 ± 5·6 pM; 15 min refed, 55·8 ± 15·6 pM; 30 min refed, 48·4 ± 15·0 pM. No difference was seen in the corpus c-*fos* response to feeding between animals treated with gastrin or control antibody (control immunoneutralized, 100 ± 16·2%; gastrin immunoneutralized, 121·5 ± 24·9%; $n = 6$). The refeeding response was not significantly affected in rats pretreated with atropine (control corpus, 100 ± 26%; atropine corpus, 126 ± 42%; control antrum, 100 ± 29%; atropine antrum, 87 ± 34%; $n = 6$). Pretreatment of animals with hexamethonium significantly reduced the corpus c-*fos* response to refeeding but it did not abolish it (control, 100 ± 20·4%; hexamethonium, 45 ± 9·5; $P < 0.05$, $n = 6$). Neither c-*jun*, c-*myc*, nor GAPDH mRNAs were elevated by refeeding fasted animals (Fig. 2).

Distension of the stomach in conscious rats prepared with gastric fistulae for 30 min with methyl cellulose produced a significant stimulation of c-*fos* mRNA ($P < 0.05$, ANOVA) similar to that seen after 30 min refeeding of fasted animals (Fig. 3). Distension with peptone produced a slightly greater response than with methyl cellulose (Fig. 3), but the difference was not statistically significant. However, gastric distension also significantly elevated mRNAs encoding c-*jun* and c-*myc*, but not GAPDH (Fig. 2).

*In situ* hybridization with a $^{35}$S-labelled antisense oligonucleotide probe to c-*fos* revealed intense labelling of gastric myenteric neurones in animals subjected to gastric distension; similar, but less intense responses were also obtained in animals refed following a fast. No labelling of gastric myenteric neurones was observed in control (fasted, undistended) animals (Fig. 4).

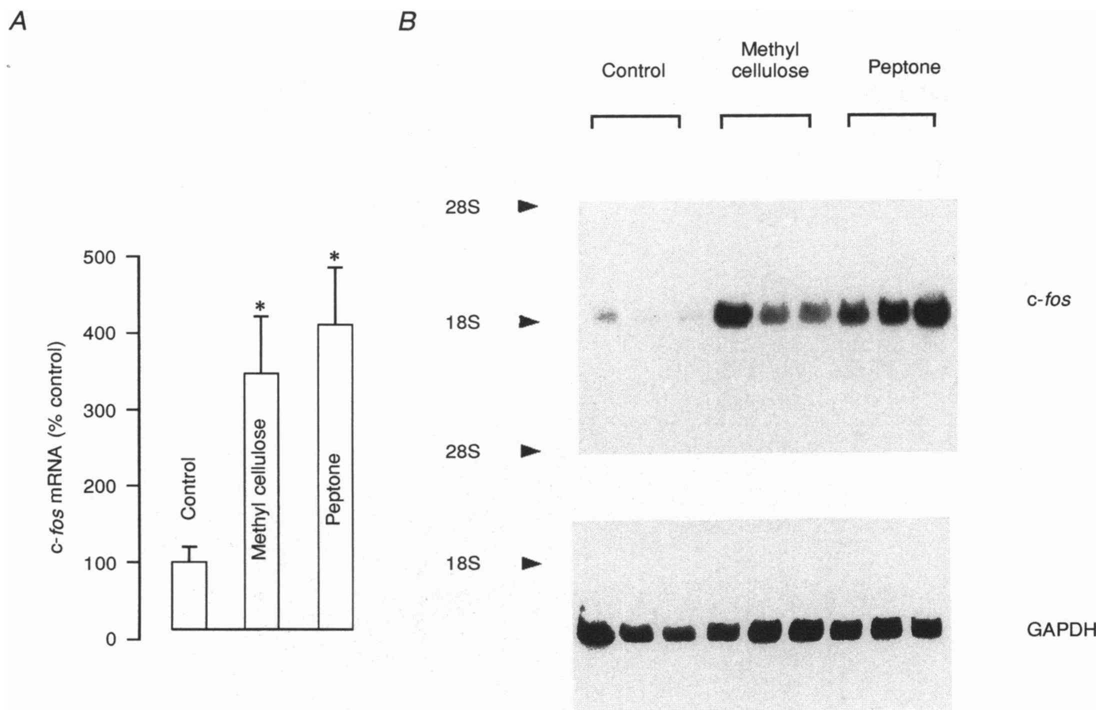


**Figure 3. Expression of c-*fos* in response to gastric distension**

*A*, c-*fos* mRNA abundance in 24 h fasted conscious gastric fistula rats that received no gastric distension (control, $n = 4$), or 30 min distension to 5 cmH$_2$O pressure with 4·5% peptone solution ($n = 6$) or 2% methyl cellulose solution ($n = 6$). Values are means ± S.E.M., * $P < 0.05$ relative to control, ANOVA. *B*, representative Northern blot of total RNA from the gastric corpus of control gastric fistula rats (lanes 1–3) or rats receiving gastric distension with methyl cellulose (lanes 4–6) or peptone (lanes 7–9). Membranes were hybridized with a c-*fos* cRNA probe (upper signal), stripped then rehybridized with a GAPDH cRNA probe (lower signal).

## DISCUSSION

The present findings demonstrate that expression of the immediate early gene c-*fos* in gastric myenteric neurones is an early response to the physiological stretching of the stomach wall that accompanies feeding. A greater than fivefold increase in gastric corpus c-*fos* mRNA was recorded 15 min after the onset of feeding following a prolonged fast; within 30 min of feeding the response had already begun to decline. The time course of the c-*fos* response to the first few hours of refeeding fasted animals is remarkably similar to that of the antral hormone gastrin seen here and in previous studies (Dimaline *et al.* 1993). To determine if gastrin might be involved in the c-*fos* response to feeding we examined c-*fos* mRNA after 30 min refeeding in rats that had been passively immunoneutralized with a monoclonal antibody specific for the biologically active amidated *C*-terminus of gastrin. The antibody was administered in a dose that has previously been shown to neutralize the acid secretory response to endogenous gastrin (Yang *et al.* 1989). For comparison, we examined the 30 min refeeding response in animals that received a monoclonal antibody to the glycine-extended biosynthetic precursor for gastrin which has no effect on acid secretion. The c-*fos* response to feeding was unaffected by immunoneutralization, as it was by pretreatment with atropine suggesting that gastrinergic or muscarinic cholinergic mechanisms do not mediate the response.

Two components of feeding that might trigger expression of immediate early genes are physical distension of the stomach and chemical stimulation of the mucosa. To

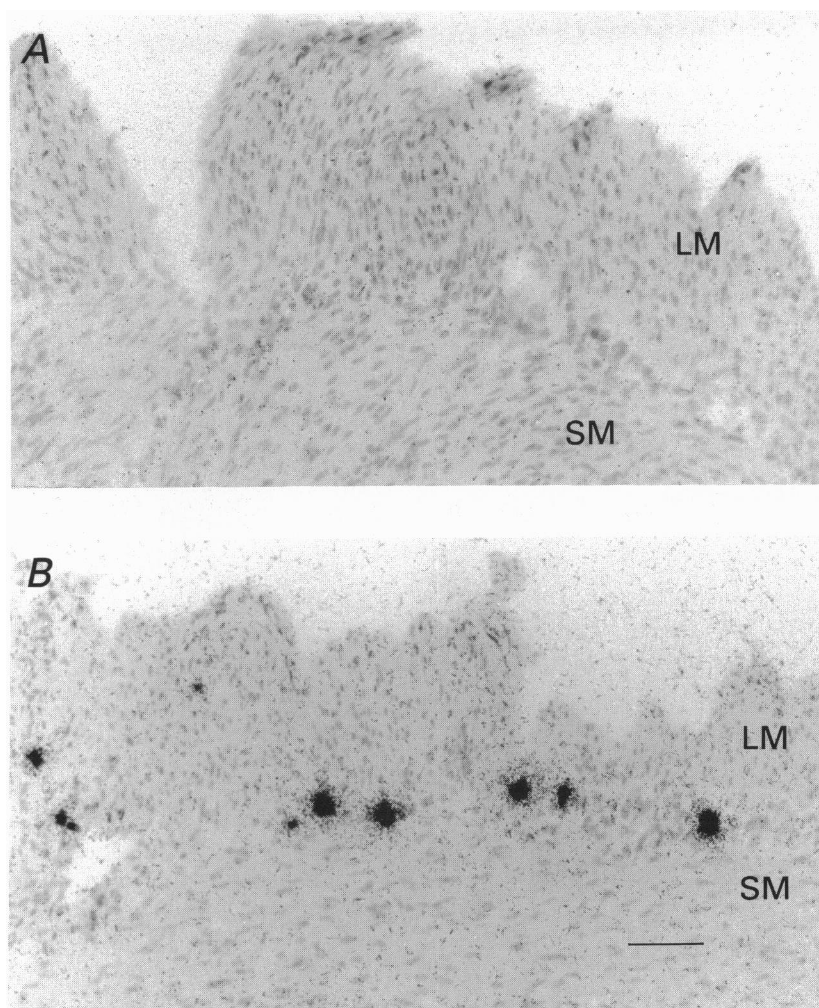


**Figure 4. *In situ* hybridization of c-*fos* mRNA in rat gastric myenteric plexus**

Sections were hybridized with a $^{35}$S-labelled antisense c-*fos* oligonucleotide. LM, longitudinal muscle; SM, circular muscle; scale bar, 20 $\mu$m. *A*, gastric fistula rats fasted for 48 h. *B*, gastric fistula rats fasted for 48 h then subjected to 30 min gastric distension with methyl cellulose at a pressure of 5 cm$H_2O$.

distinguish between these possibilities we distended the stomachs of gastric fistula rats at constant pressure (5 $cmH_2O$) with either peptone, to simulate a meal, or methyl cellulose which increases viscosity in the absence of chemical stimulation. Previous studies have shown that a distending pressure of 5 $cmH_2O$ is well within the physiological range of rat intragastric pressures (Raybould, Dockray & Gayton, 1985; Dimaline *et al.* 1986). Both distension protocols elicited c-*fos* expression similar to that seen after 30 min feeding, suggesting that passive distension of the stomach alone could account for the response.

In contrast, gastric distension, but not feeding, induced expression of c-*jun* and c-*myc*. A likely explanation for this diversity of response is that during feeding the stomach relaxes to accommodate the meal, thereby reducing tension in the stomach wall. During distension via a gastric fistula a pressure of 5 $cmH_2O$ (albeit within the physiological range) is constantly applied for 30 min. Although the stomach relaxes to accommodate this simulated meal, the pressure is unable to fall below 5 $cmH_2O$. The possibility cannot be excluded, however, that c-*myc* and c-*jun* are elevated later than 30 min in the normal response to feeding. Immunohistochemical studies have demonstrated c-*myc* protein in intestinal myenteric neurones of unstimulated animals (Parr, Gibson & Sharkey, 1994). The present findings failed to show increases in gastric c-*myc* in response to feeding so that increased expression of c-*jun* and c-*myc* might therefore be considered responses to supraphysiological stretching of the stomach wall.

Previous immunohistochemical studies (Fraser & Davison, 1993) indicate that feeding a liquid meal to fasted rats elicits expression of c-*fos*-like immunoreactivity in brainstem neurones present in the same region as cells that alter their firing rate in response to gastric distension (Raybould *et al.* 1988). The response in this case is thought to be mediated by mechanoreceptors on vagal afferent fibres, whose cell bodies lie in the nodose ganglion. Increases in immediate early gene mRNA abundance have also been demonstrated in mucosal scrapings from rat small and large intestine within one to four hours of refeeding fasted animals (Holt & Du Bois, 1991; Hodin, Graham, Meng & Upton, 1994). These immediate early gene responses were interpreted as the mitogenic response to feeding within the crypt compartment of the intestinal epithelium, although the precise cellular localization was not determined. Expression of c-*fos*-like immunoreactivity in guinea-pig small intestinal enteric neurones has, however, been demonstrated in response to application of pressure or cholera toxin to the mucosa and the responses were diminished but not abolished by hexamethonium (Kirchgessner, Tamir & Gershon, 1992). The cells that continued to express c-*fos* in the presence of hexamethonium were presumed to be primary afferent (intrinsic) neurones. In the present study, c-*fos* mRNA has been localized to cell bodies of gastric myenteric neurones. Since it is known that intrinsic myenteric neurones are also able to act as mechanoreceptors, and that c-*fos* expression is a useful marker of neuronal activity (Hunt, Pini & Evan, 1987; Sagar, Sharp & Curran, 1988), the most economical explanation for the present findings is that the residual c-*fos* expression in the presence of hexamethonium occurs in neurones responding directly to mechanical deformation. The hexamethonium-sensitive c-*fos* expression in response to feeding presumably occurs in cells that are activated by ganglionic transmission. The long-term cellular events initiated by c-*fos* expression in the gastric myenteric plexus remain to be determined.


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