Spatial localization and properties of pacemaker potentials in the canine rectoanal region

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Mutafova-Yambolieva, V. N., K. O’Driscoll, A. Farrelly, S. M. Ward, and K. D. Keef. Spatial localization and properties of pacemaker potentials in the canine rectoanal region. Am J Physiol Gastrointest Liver Physiol 284: G748–G755, 2003. First published January 22, 2003; 10.1152/ajpgi.00295.2002.—The present study investigated the spatial organization of electrical activity in the canine rectoanal region and its relationship to motility patterns. Contraction and resting membrane potential (\(E_m\)) were measured from strips of circular muscle isolated 0.5–8 cm from the anal verge. Rapid frequency [25 cycles/min (cpm)] \(E_m\) oscillations (MPOs, 12 mV amplitude) were present across the thickness of the internal anal sphincter (IAS; 0.5 cm) and \(E_m\) was constant (−52 mV). Between the IAS and the proximal rectum an 18 mV gradient in \(E_m\) developed across the muscle thickness with the submucosal edge at −70 mV and MPOs were replaced with slow waves (20 mV amplitude, 6 cpm). Slow waves were of greatest amplitude at the submucosal edge. Nifedipine (1 μM) abolished MPOs but not slow waves. Contractile frequency changes were commensurate with the changes in pacemaker frequency. Our results suggest that changing motility patterns in the rectoanal region are associated with differences in the characteristics of pacemaker potentials as well as differences in the sites from which these potentials emanate.

THE RECTOANAL REGION REPRESENTS the final site for controlling the storage, transport, and evacuation of gastrointestinal contents. The internal anal sphincter (IAS) is a thickening of the circular muscle layer at the distal end of the rectum. Under most circumstances, the IAS is closed and contributes to maintaining continence of fecal matter, liquids, and gases, whereas during the defecation reflex, it briefly relaxes to allow the passage of fecal matter. In contrast, although the rectum is generally empty, it can serve as a final site of storage before defecation as well as participating in the defecation reflex. The IAS and rectum are, therefore, anatomically linked but subserve different physiological roles (7, 13, 16, 18). These functional differences are likely to be associated with significant differences in the motility patterns in the two regions as well as the mechanisms controlling this activity.

Spontaneous phasic contractile activity in gastrointestinal smooth muscle is generally associated with pacemaker potentials of varying time course and amplitude. In recent years, there has been mounting evidence that this activity is generated by specialized cells referred to as interstitial cells of Cajal (ICC) (14). In the human and canine colonic circular muscle layer, for example, slow waves arise from a specific population of ICC located at the submucosal edge of the muscle layer, and these give rise to a characteristic 1-cpm (4) or 6-cpm contractile rhythm (17), respectively. In contrast, although the IAS exhibits spontaneous contractile activity (8), much less is known about the mechanisms underlying this activity or the role that ICC play in this process.

The goal of the present study was to characterize pacemaker potentials of the canine rectoanal region over an 8-cm distance from distal IAS to proximal rectum and to correlate this with the spontaneous contractile activity occurring in this region. Special attention was given to determining the spatial characteristics of electrical activity in an effort to predict the sites from which pacemaker potentials may arise. Our results suggest that the characteristics of pacemaker potentials and the site from which they emanate changes from IAS to rectum. In Ref. 2 the anatomic distribution of putative pacemaker cells is examined.

MATERIALS AND METHODS

Mongrel dogs of either sex (0.5–2 yr of age) were killed with an overdose of pentobarbital sodium (100 mg/kg) administered into the femoral vein. The pelvic rectoanal region was exposed by sawing through the midline of the pelvic bone. Incisions were made on either side of the rectum and through the skin adjacent to the anus to allow removal of the last 10- to 12-cm portion of the gastrointestinal tract. The dissected segment was cut open from anus to proximal rectum and fecal material was removed. All adhering skeletal muscle and glands were then removed after pinning the segment in a dissecting dish. Krebs bicarbonate solution of the following composition was used (in mM) 118.5 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 23.8 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), and 11.0 dextrose. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O\(_2\)-5% CO\(_2\). All experiments, unless otherwise stated, were performed in the presence of 1 μM atropine, 1 μM phentolamine, and 100 μM NO-\(\text{-nitro-L-arginine methylester}\) (L-NAME).
nine (L-NNA) to remove the potential contributions of cholinergic, adrenergic, and nitricergic nerves.

The rectoanal region was pinned taut (but not overtly stretched) in a dissecting dish. The distal-most extension of the IAS was identified and is referred to in this study as the anal verge. Two-millimeter-wide strips of the tunica muscularis were created at various distances from the anal verge by cutting the tissue parallel to the circular muscle fibers with a knife consisting of a pair of parallel scalpel blades set 2 mm apart. The rectoanal lengths in vivo are likely to be slightly less than those reported here, because the muscle in vivo is untethered. All of the mucosa and most of the underlying submucosa was removed from strips by sharp dissection leaving behind enough connective tissue to pin the preparation in place and ensure the integrity of cells at the submucosal edge of the circular muscle layer. For contractile experiments whole muscle strips isolated at 1, 2, 4, and 8 cm from the anal verge were attached with sutures to a stable mount and to a Gould strain gauge and immersed in tissue baths containing 10 ml Krebs bicarbonate solution oxygenated and maintained at 37°C. A basal tension of 1 g was applied to muscle that was dissected 0–30 min before the tissue used. The applied tension declined to ~0.2–0.3 g (as assessed from periods of minimum spontaneous rhythmic contraction). Contractile patterns were evaluated once baseline tension had stabilized.

For intracellular measurements, muscle strips isolated 0.5, 3, 5, and 8 cm from the anal verge were pinned cross-sectional to the floor of an electrophysiological chamber. In this way, the entire thickness of the circular muscle layer could be visualized, making it possible to record from specific positions within the muscle layer. The position of each impalement within the circular muscle layer has been expressed as the %distance from the submucosal edge. In some experiments, the circular muscle of the IAS and proximal rectum were further divided into sections by cutting through the muscle layer parallel to the circular muscle fibers by using a microsurgical knife. Muscle cells were impaled with glass microelectrodes filled with 3 M KCl and having resistances ranging from 40 to 80 MΩ. Impalements were accepted on the basis of previously discussed criteria (15).

Membrane potential (Em) was measured with a high input impedance electrometer (model Duol 773; World Precision Instruments), and outputs were displayed on an oscilloscope (model 3091; Nicolet). Analog electrical signals were digitized and recorded on video tape (model 875; Vetter). Data were also stored and analyzed by computer by using a data acquisition program (AcqKnowledge; Biopac Systems).

Analysis of data. Several different parameters of electrical activity were tabulated. Resting Em of cells was determined as the most probable potential attained between membrane potential oscillations (MPOs) in the IAS or slow waves in the rectal region. Slow-wave amplitude was determined as the peak level of depolarization attained during the plateau phase. In cases where both MPOs and slow waves were recorded at the myenteric edge of the rectum, only the slow-wave frequency was used to determine pacemaker potential frequency. In addition, when slow waves were very small in amplitude at this edge, slow-wave parameters were measured from those portions of the recording in which slow waves were most readily distinguishable.

Because the tone of the IAS fluctuated, contractile amplitude was determined by averaging the peak contractions achieved during a 20-min time period (3–6 peaks). Basal tension was taken as the minimum level of tension occurring during this period of time. Contractile amplitude therefore included both the slow fluctuation in tone plus the small rapid superimposed contractions. Spontaneous contractile amplitude in rectal segments was determined as the average of the five largest phasic contractions occurring during a 20-min period of time. It was not possible to elicit a maximum agonist contraction in the same tissue in which spontaneous contractions were measured because these tissues were continually bathed in adrenergic and cholinergic antagonists. Furthermore, the addition of high concentrations of norepinephrine in the absence of blockers produces changes in the spontaneous contractile activity of the IAS that persists for hours. Thus maximum contractile amplitude was determined in nine additional tissues from each region that had not been exposed to blockers. Norepinephrine (100 µM) was used for IAS strips (1 and 2 cm) and 1 mM acetylcholine was used for rectal strips (4 and 8 cm), because these agonists and concentrations produce maximum contractions (21). Spontaneous contractile responses were then normalized to these mean maximum responses.

Significant differences between means in the four rectoanal regions was determined by using one-way ANOVA followed by a Tukey-Kramer multiple comparisons test. Means were considered significantly different when P < 0.05. Only one muscle strip per rectoanal region was used from any one animal; thus n values represent both the number of animals and the number of muscle strips used.

**Drugs.** Tetrodotoxin, atropine sulfate, phentolamine, L-NNA, acetylcholine, norepinephrine, and nifedipine were all purchased from Sigma (St. Louis, MO). Nifedipine was dissolved in ethanol. Other drugs were dissolved in distilled water.

**RESULTS**

Anatomic characterization of the rectoanal region. The gross morphologic features of the rectoanal region were examined at the light level by cutting a thin strip of muscle perpendicular to the circular muscle fibers from the distal IAS to the proximal rectum. Strips were then pinned cross-sectional to reveal the circular and longitudinal muscle layers throughout this region. In the rectum, a thin, densely packed, circular muscle layer was apparent. The longitudinal muscle layer was of approximately the same thickness as the circular muscle layer and was separated from it by a thin connective tissue space (Fig. 1). In the distal direction, there was a progressive widening of the separation between longitudinal and circular muscle layers. At the level of the IAS the longitudinal muscle layer dissipated, no longer forming a discrete structure. In contrast, the circular muscle layer becomes thicker in the distal direction, particularly within the last 2-cm section. There was also a clear change in the density of muscle fibers from rectum to IAS with the rectum being compact and dense, whereas the IAS was diffuse and separated into bundles (Fig. 1).

Although cutting the tissue perpendicular to the circular muscle fibers was useful for visualizing the entire rectoanal structure, all contractile and electrophysiological experiments were carried out on segments cut parallel to the circular muscle fibers. The entire muscle segment used to create muscle strips is shown diagrammatically in Fig. 2. For most experiments, muscle strips isolated at four different distances from the anal verge were used. The insets depict how in some experiments, muscle strips from the IAS...
(1 cm) and the rectum (8 cm) were further divided into subregions.

Electricity activity in the IAS and rectum. $E_m$ was recorded from strips of muscle isolated from various sites 0.5–8 cm from the anal verge. Muscle strips were oriented so that the entire muscle thickness was visible and recordings were made at 5, 50, and 95% distance from the submucosal edge. In the IAS (0.5 cm) there was no significant difference in the level of resting $E_m$ across the thickness of the muscle layer (Fig. 3A). In contrast, an 18-mV gradient in $E_m$ was observed from the submucosal edge ($-70.2 \pm 3$ mV) to the myenteric edge ($-51.8 \pm 3.2$ mV) of the proximal rectum (8 cm). Between these two extremes, there was a gradual development of more negative potentials at the submucosal edge, whereas the myenteric edge remained unchanged ($n = 4–6$; Fig. 3A).

Rapid-frequency MPOs averaging 10–11 mV in amplitude and having a frequency of 20–25 cpm were recorded at 5, 50, and 95% distance from the submucosal edge of the IAS (0.5 cm). There was no significant difference in the amplitude or frequency of MPOs at these three positions (Fig. 3, B and C). Addition of tetrodotoxin (1 $\mu$M) in the continued presence of atropine (1 $\mu$M), phentolamine (1 $\mu$M), and L-NNA (100 $\mu$M) did not change the time course or amplitude of MPOs recorded in the middle of these strips ($n = 3$). To further explore the origin of MPOs, additional experiments were undertaken in which the IAS was subdivided into sections, i.e., myenteric, interior, and submucosal (see Fig. 2). MPOs were observed in each of these three subsections (Fig. 4A) and their amplitudes were not significantly different from one another ($n = 5$) (Fig. 4B).

In the rectum (8 cm), slow waves were present and these were of greatest amplitude at the submucosal edge ($20 \pm 4$ mV amplitude, 6 cpm; $n = 6$) and declined with distance away from this edge (Figs. 3B and 5) suggesting that they arise from cells near the submucosal edge. Addition of tetrodotoxin (1 $\mu$M) in the continued presence of atropine (1 $\mu$M), phentolamine (1 $\mu$M), and L-NNA (100 $\mu$M) did not change the time course or amplitude of slow waves recorded at the submucosal edge ($n = 3$). To further explore the origin of slow waves, the rectal muscle layer was divided into two sections i.e., a myenteric and a submucosal half (see Fig. 2). Rectal strips were divided into two rather than three sections, because the muscle layer in this region is less than half the thickness of the IAS and because the intent was to establish from which one-half of the muscle layer slow waves originate. Recordings in submucosal strips were made at the 5% position and those in myenteric strips were made at the 95% position. Slow waves averaging $23.8 \pm 3.7$ mV in amplitude and $5.0 \pm 0.8$ cpm in frequency were observed in submucosal strips ($n = 7$), whereas six of seven myenteric strips exhibited MPOs that were much smaller and irregular in appearance ($4.5 \pm 1.0$ mV amplitude and $16.9 \pm 3.5$ cpm frequency, $n = 6$) (Fig. 6). One myenteric strip differed in that it had extremely small (i.e., 1 mV) slow waves occurring at a frequency of 4 cpm.

Effect of nifedipine on electrical activity. Nifedipine is often used as a diagnostic tool to evaluate whether an electrical oscillation is generated by ICC, because in most cases, pacemaker potentials are resistant to dihydropyridines (24). The ability of nifedipine to inhibit pacemaker potentials in strips of IAS (1 cm) and rec-

Fig. 1. Gross anatomy of the internal anal sphincter (IAS) and rectum. A: low-power image of an unstained 3-mm wide strip of muscle cut parallel to the longitudinal muscle fibers from IAS to rectum. The circular muscle layer is highlighted with a broken white line. Running parallel and above the circular muscle layer is the longitudinal muscle layer separated from it by a connective tissue space. The heavy dark band above the area marked cm is actually the myenteric surface viewed on side, because the tissue is oriented somewhat obliquely. B: higher-power view of the sphincter region labeled in A with an asterisk (left). Note the feather-like appearance of the circular muscle (cm) layer in this region due to the presence of muscle bundles separated by connective tissue septa. The heavy dark band above the area marked cm is actually the myenteric surface viewed on side, because the tissue is oriented somewhat obliquely. C: higher-power view of the rectal region labeled in A with an asterisk (right). Note the thinner, more compact appearance of this portion of the circular muscle. A single septal structure is apparent that traverses the muscle layer. A thin connective tissue layer is interposed between longitudinal muscle (lm) and circular muscle.
tum (8 cm) was therefore tested. Nifedipine (1 μM) entirely abolished MPOs recorded near either the myenteric (95%) or submucosal edge (5%) of the IAS as seen in Fig. 7A (n = 4). There was no significant change in the value of mean resting $E_m$ ($-51 \pm 2$ vs. $-50 \pm 4$ mV, $n = 4$). In contrast to the IAS, the area of depolarization occurring during the rectal slow wave (recorded 5% from the submucosal edge) was only reduced by 51.5 $\pm$ 13% in six of eight tissues, whereas in two tissues, slow waves were abolished. The reduction in area was predominantly due to a reduction in the plateau phase of the slow wave (Fig. 7B). Nifedipine did not significantly change the rectal resting $E_m$ ($-71 \pm 2$ vs. $-69 \pm 3$ mV, $n = 8$).

Contractile activity in the rectoanal region. All muscle strips (1–8 cm; $n = 40$) exhibited spontaneous contractile activity. Examples of this activity are shown in Fig. 8A. The contractile pattern in the IAS was complex and consisted of a slow fluctuating level of tone with superimposed high frequency contractions that averaged 19 $\pm$ 1.2 cpm at 1 cm (Fig. 8B). In contrast, baseline tension between contractions in the rectum remained constant. Contractile frequency declined in the proximal direction and averaged 5.7 $\pm$ 0.7 cpm in the 8 cm muscle strips (Fig. 8B). There was no significant difference in the amplitude of spontaneous contractions between muscle regions when raw contractile amplitudes were tabulated (Fig. 8C). However, maximum contractile amplitude significantly increased in the proximal direction although each strip was cut to the same width (Fig. 8D). This difference likely reflects the greater quantity of connective tissue present distally (2). When spontaneous contractions were normalized to the maximum contraction, there was a significant decline in the proximal direction (Fig. 8E).

DISCUSSION

The rectum and IAS are adjacent structures that are anatomically linked but subserve different physiological roles. These differences are associated with changes
in the motility patterns in progressively more distal segments including an increase in contractile frequency accompanied by an increase in the frequency of pacemaker potentials. A gradient in $E_m$ also exists across the thickness of the rectum with an apparent submucosal site of origin for slow waves. In the distal direction the electrical gradient becomes normalized and pacemaker potentials appear to emanate from throughout the muscle layer. The significance of these changes with regard to the mechanisms responsible for generating pacemaker potentials are discussed below.

Spontaneous phasic contractions and oscillations in $E_m$ occurred in all muscle strips isolated from the rectoanal region. However, characteristics of both changed significantly in the distal direction. The slow waves of the proximal rectum were very similar in time course to those previously described in the canine colon (17). And like colonic slow waves (24), rectal slow waves were not blocked by nifedipine. Several studies have suggested that slow waves in the canine colon arise from ICC located at the submucosal edge of the circular muscle layer (9, 12, 17, 22, 23, 25). These studies have shown that 1) a plexus of ICC exists at the submucosal edge; 2) slow waves can be recorded from ICC enzymatically dispersed from this edge by using the patch clamp technique; 3) in the intact tissue, slow waves are of greatest amplitude along the submucosal edge and decline almost to zero at the myenteric edge; and 4) slow waves are absent from the bulk smooth muscle when a thin strip of muscle containing the submucosal edge is removed from the tissue or is chemically damaged.

Our studies in the canine rectum also revealed a gradient in slow-wave amplitude across the thickness of the circular muscle layer. Furthermore, we found that when the muscle layer was divided into submucosal and myenteric halves that slow waves occurred only in the submucosal half. Thus rectal slow waves, like those of the colon, are likely to be generated by submucosal ICC. Rectal slow waves differ in that their amplitude is only ~50% of those in the colon. In more distal segments, the amplitude of pacemaker potentials declines even further. Despite this, the amplitude of spontaneous contractions throughout the rectoanal region is relatively constant (see Fig. 8). This situation may result because of the concurrent, reduced polarization of resting $E_m$ in the distal direction. A reduced resting $E_m$ would ensure that smaller amplitude oscillations still exceed the electrical threshold (19) for contraction. In contrast to rectal slow waves, there was no gradient in the amplitude of MPOs across the thickness of the IAS. Furthermore, when the IAS was subdivided (i.e., myenteric, interior, and submucosal sections) each subsection exhibited MPOs of equal amplitude. Thus unlike the rectum, no distinct site of origin was apparent for MPOs, but rather, they appeared to arise from pacemaker cells located throughout the circular muscle layer.

Because MPOs were abolished by the L-type calcium channel blocker nifedipine, one might argue that they must be of smooth muscle origin. Patch clamp studies in recent years suggest that the pacemaker currents of...
ICC are due to nonselective cation channels that are insensitive to dihydropyridines (6, 20). However, these studies involved the use of cultured ICC that may lead to downregulation of calcium channel activity. In fact, in one of the few patch clamp studies (10) completed using freshly dispersed ICC, it was reported that both L-type and T-type calcium channel currents were present. Interestingly, the MPOs recorded from the IAS in our study were indistinguishable in time course, amplitude, and frequency from the MPOs previously described for the myenteric edge of the canine colonic circular muscle layer (17). Previous studies have suggested that canine colonic MPOs arise from ICC located at the myenteric edge because: 1) a plexus of ICC is present at this edge (22), 2) colonic MPOs are of greatest amplitude in the vicinity of the ICC plexus and decline with distance away from this region (17), and 3) MPOs are absent from muscle strips that lack the myenteric edge (3). A further resemblance between colonic and sphincteric MPOs is that both are abolished by nifedipine (Ref. 3 and present study). Given these similarities it is tempting to speculate that the MPOs generated in both regions are due to a specific subset of ICC more highly dependent on L-type calcium channel activity for the generation and/or the conduction of pacemaker potentials to the adjacent smooth muscle. Because MPOs in the IAS are recorded throughout the muscle layer and in subsegments of this layer, we would predict that ICC are likely to be diffusely distributed rather than being restricted to a dense plexus region at one or the other edge.

Fig. 6. Electrical activity recorded in two subregions of the rectal circular muscle layer (8 cm). Muscle strips were created as shown in Fig. 2. In the submucosal half of the circular muscle layer (top trace) slow waves were observed, whereas in the myenteric half, MPOs are apparent (bottom trace). Slow waves were not observed in strips of the myenteric half of the muscle.

Fig. 7. Effect of nifedipine on MPOs and slow waves in the rectoanal region. A: continuous recording from a cell in the IAS (1 cm). MPOs are present that were abolished after superfusion of the tissue with nifedipine (1 μM). B: continuous recording from a cell near the submucosal edge of the circular muscle layer in the rectum (8 cm). Slow waves were reduced in duration and in upstroke velocity by nifedipine, but they were not abolished. There was also an increase in slow wave frequency.
Our study suggests that the transition from IAS to rectum is gradual rather than abrupt. This is true both anatomically in that one sees a gradual reduction in circular muscle thickness in the proximal direction as well as a gradual increased polarization of $E_m$ at the submucosal edge. On the other hand, one can loosely describe the sphincter and its transition into rectum as encompassing a 3-cm region of the distal GI tract, because over this distance, pacemaker potentials decline from 25 cpm at 0.5 cm to the rectal/colonic frequency of 5–6 cpm at 3 cm. In a related study, we have also found that motor innervation at 1 cm is exclusively sympathetic (i.e., abolished by guanethidine), whereas it constitutes ~80% of the neural response at 2 cm and 20% at 4 cm (21).

In the IAS, a complex pattern of spontaneous contractile activity was observed consisting of slow fluctuations in tone with superimposed contractions occurring at the MPO frequency. Intracellular recordings throughout the muscle layer revealed MPO activity. A previous study (8) of this region reported that each rapid frequency contraction was associated with an electrical event was described for slow fluctuations in tone. In our study, electrical and contractile activity were recorded from different preparations; thus we cannot be certain as to the origin of the slow fluctuations in tone. It is likely that MPOs produce summed contractions as well as high-frequency phasic contractions. The tendency of the muscle to produce summed contractions is greatly enhanced with low frequency sympathetic nerve stimulation, e.g., even 1-Hz nerve stimulation of the canine IAS generates a tonic contracture (21). Spontaneous contractile amplitude of IAS muscle strips is far below what the muscle is capable of generating (i.e., 12.5% of maximum at 1 cm, see Fig. 8). In contrast, 20 Hz nerve stimulation produces an 85% maximum contraction of the same muscle strips (21). Thus it is not surprising that removal of sympathetic neural input to the IAS in vivo leads to a substantial decline in sphincteric tone (e.g., see Refs. 1 and 13). In summary, the function of the IAS (i.e., to generate tone to aid in maintaining continence) is likely to be achieved through the combined actions of sympathetic nerves in concert with the inherent tendency of this tissue to produce ongoing high-frequency electrical oscillations.

The pattern of spontaneous contractions in the canine rectum and the underlying slow waves are very similar to the contractile and electrical activity of the canine colon. However, the amplitude of spontaneous rectal contractions is considerably larger, i.e., rectal muscles generate spontaneous contractions that are 10 and 7% of maximum at 4 and 8 cm, respectively, whereas spontaneous contractions of the canine colon are generally <1% of maximum (5). Whereas the colon has a reabsorptive role, the rectum usually remains empty via nonpropulsive segmentation. Greater spontaneous contractile activity in this region would aid in this function.

The development of an electrical gradient across the thickness of the circular muscle layer from IAS to rectum is both novel and interesting. This transition occurs because the submucosal edge becomes progressively more negative, whereas the myenteric edge remains at the same potential. Associated with the development of an electrical gradient is the transformation of pacemaker potentials from MPOs to slow waves along with an apparent change in the site of
ICC AND PACEMAKER POTENTIALS IN RECTOANAL REGION

origin of pacemaker potentials. Previous studies (11) of the proximal colon suggest that submucosal ICC are responsible for drawing the $E_m$ in this region down to more negative levels. Thus the appearance of slow waves in the rectum is likely to be correlated with the appearance of a discrete submucosal plexus of ICC. In Ref. 2 the anatomic characteristics of ICC and their relationship to smooth muscle and nerves in the canine rectoanal region are explored.

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