CRF-induced calcium signaling in guinea pig small intestine myenteric neurons involves CRF-1 receptors and activation of voltage-sensitive calcium channels

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Bisschops, R., P. Vanden Berghe, G. Sarnelli, J. Janssens, and J. Tack. CRF-induced calcium signaling in guinea pig small intestine myenteric neurons involves CRF-1 receptor activation and voltage-sensitive calcium channels. Am J Physiol Gastrointest Liver Physiol 290: G1252–G1260, 2006. First published December 29, 2005; doi:10.1152/ajpgi.00349.2004.—Corticotropin-releasing factor (CRF) is a 41-amino acid peptide with distinct effects on gastrointestinal motility involving both CRF-1 and CRF-2 receptor-mediated mechanisms that are generally claimed to be centrally mediated. Evidence for a direct peripheral effect is rather limited. Electrophysiological studies showed a cAMP-dependent prolonged depolarization of guinea pig myenteric neurons on application of CRF. The current study aimed to test the direct effect of CRF on myenteric neurons and to identify the receptor subtype and the possible mechanisms involved. Longitudinal muscle myenteric plexus preparations and myenteric neuron cultures of guinea pig small intestine were incubated with the calcium indicator Fluo-4. Confocal Ca²⁺ imaging was used to visualize activation of neurons on application of CRF. All in situ experiments were performed in the presence of nicardipine 10⁻⁶ M to reduce tissue movement. Images were analyzed using Scion image and a specifically developed macro to correct for residual minimal movements. A 75 mM K⁺–Krebs solution identified 1,076 neurons in 46 myenteric ganglia (16 animals). Administration of CRF 10⁻⁶ M and CRF 10⁻⁷ M during 30 s induced a Ca²⁺ response in 22.4% of the myenteric neurons (n = 303). Responses were completely abolished in the presence of the nonselective CRF antagonist astressin (n = 55). The selective CRF-1 receptor antagonist CP 154,526 (n = 187) reduced the response significantly to 2.1%. Stresscopin, a CRF-2 receptor agonist, could not activate neurons at 10⁻⁷ M, and its effect at 10⁻⁶ M (15.3%, n = 59) was completely blocked by CP 154,526. TTX 10⁻⁶ M (n = 70) could not block the CRF-induced Ca²⁺ transients but reduced the amplitude of the signals significantly. Removal of extracellular Ca²⁺ blocked all responses to CRF (n = 47). L-type channels did not contribute to the CRF-induced Ca²⁺ transients. Blocking N- or P/Q-type Ca²⁺ channels did not reduce the responses significantly. Combined L- and R-type Ca²⁺ channel blocking (SNX-482 10⁻⁸ M, n = 64) abolished nearly all responses in situ. Combined L-, N-, and P/Q-type channel blocking also significantly reduced the response to 8.6%. Immunohistochemical staining for CRF-1 receptors clearly labeled individual cell bodies in the ganglia, whereas the CRF-2 receptor staining was barely above background. CRF induces Ca²⁺ transients in myenteric neurons via a CRF-1 receptor-dependent mechanism. These Ca²⁺ transients highly depend on somatic calcium influx through voltage-operated Ca²⁺ channels, in particular R-type channels. Action potential firing through voltage-sensitive sodium channels increases the amplitude of the Ca²⁺ signals. Besides centrally mediated effects, CRF is likely to modulate gastrointestinal motility on the myenteric neuronal level.

CORTICOTROPIN-RELEASING-FACTOR (CRF) is a 41-amino acid peptide with distinct effects on gastrointestinal motility both in humans and in animals. CRF exerts its action through activation of membrane-bound Gs protein-coupled receptors (27). Until now, two distinct receptors subtypes (CRF-1 and -2) are cloned from rat and human brain (7, 12, 14, 26), and different intracellular signaling pathways have been demonstrated depending on the tissue or cell lines studied (9).

Several studies have shown effects of CRF on gastrointestinal motility, both through central and peripheral mechanisms. In humans, intravenous administration of CRF was shown to stimulate pyloric and duodenal motility (32). The effect of CRF on gastrointestinal motility of rats has been studied in more detail and appears to involve both types of receptors exerting different effects depending on their localization in the gastrointestinal tract. Peripherally administrated CRF inhibits gastric emptying via CRF-2 receptor activation and stimulates colonic motility and induces diarrhea via CRF-1 receptor-mediated mechanisms (29, 21, 17, 24, 25). Delayed gastric emptying is thought to be the main mechanism behind the anorexic effect of CRF, exerted via CRF-2 receptor activation. Recent reports (16), however, also mention a CRF-1-dependent central mechanism in postoperative gastric ileus. Although CRF-2- and CRF-1-mediated effects were respectively attributed to central vagal and extrinsic orthosympathetic pathways (1, 8, 18, 20), peripheral modes of action cannot be excluded. In rats, CRF has a direct excitatory action on colonic motility, which was tetrodotoxin TTX sensitive and therefore neuronal in origin (19). In guinea pig small intestine, electrophysiological studies demonstrated a CRF-induced cAMP-dependent prolonged depolarization in about half of the myenteric neurons (11). It was not established whether this direct neuronal effect was mediated by activation of CRF-1 or CRF-2 receptors, and neurochemical coding of CRF-responsive neurons was not determined.

We have previously used confocal calcium (Ca²⁺)-imaging techniques to investigate ligand-induced neuronal activation in myenteric neurons in culture and in situ. (34, 35). Because the CRF-receptor activation causes a depolarization, with subse-
quent opening of voltage-dependent Ca\textsuperscript{2+} channels and secondary Ca\textsuperscript{2+} release from ryanodine-sensitive intracellular Ca\textsuperscript{2+} stores, confocal calcium imaging seems a suitable method to monitor CRF-induced neuronal activation.

The aim of the present study was therefore to investigate the direct effect of CRF administration on myenteric neurons in situ, using confocal calcium imaging. We also aimed at elucidating the involvement of CRF-1 and CRF-2 receptor activation by means of specific antagonists and agonists. Finally, we set out to identify CRF-responsive neurons immunohistochemically.

**MATERIALS AND METHODS**

**Tissue Preparation and Confocal Ca\textsuperscript{2+} Imaging**

Guinea pigs of either sex (250–700 g) were killed by cervical dislocation and exsanguinated by severing the carotid arteries, a method approved of by the Animal Ethics Committee of the University K.U. Leuven.

A portion of the proximal jejunum (the first 15 cm after the angle of Treitz) was removed and subsequently pinned out in a Sylgard-lined Petri dish to be dissected into a longitudinal muscle-myenteric plexus preparation (LMMP). Dissection was performed under continuous superfusion of a Krebs solution (4°C), which itself was continuously perfused with 95% O\textsubscript{2}-5% CO\textsubscript{2} to keep the pH at 7.4. Tissue samples of ~2.5 cm long and 1.5-cm wide were prepared and pinned out in a smaller Sylgard-lined Petri dish for bulk loading with the fluorescent calcium indicator Fluo-4 AM (9 × 10\textsuperscript{-6} M), for 45 min, at 22°C, in a tissue-specific medium [Ham’s F-12, pluronic acid 0.02% (vol/wt)], perfused with 95% O\textsubscript{2}-5% CO\textsubscript{2}. After washout with a fresh Krebs solution, a recovery period of at least 60 min was allowed, during which the tissues were continuously perfused with oxygenated Krebs at 4°C. The tissues were then stretched out over a metal ring, over which a smooth rubber ring was placed to obtain tissue immobilization.

Tissues were subsequently transferred to a coverglass bottom chamber, which was mounted on an inverted confocal scanning microscope (Nikon TE 300-Noran Oz). CRF receptor ligands and other pharmacological agents were applied by superfusion. Images of Treitz) were recorded at 2.5 Hz with a spatial resolution of 512 x 512 x 480 pixels.

**Immunohistochemical Staining**

**Immunohistochemical staining of CRF-responsive neurons.** After stimulation with CRF, tissues were incubated for 2 h or overnight in freshly prepared paraformaldehyde (4%). After being washed in 0.1 M PBS, tissues were processed for permeabilization and blocking of nonspecific binding sites by a 2-h treatment in 0.1 M PBS with Triton X-100 and 4% goat serum. Subsequently, the tissues were exposed to primary antibodies Calbindin mouse IgG MC (1/100; Sigma Immunochemicals), Calretinin Rabbit IgG PC (1/5,000; Chemicon International), Chat Rabbit IgG (1/2,000; M. Schemann, Germany), which were also diluted in the blocking medium, for a period of 48 h at 4°C. After incubation, tissues were rinsed in 0.1 M PBS (3 × 10 min) and subsequently incubated at room temperature for 60 min with the secondary antibody, diluted in the blocking medium [goat anti-rabbit Alexa Fluor 488 (1/500; Molecular Probes), goat anti-mouse carbamoylmethylindocarboyanin (Cy\textsuperscript{3}; 1/500); Jackson Immuno Research Laboratories).

Fluorescence was visualized on a Nikon Eclipse E600 microscope (excitation filters Nikon 510–560 and Nikon 470–490). Pictures were taken with an Olympus C-3040 digital camera.

**Cell Culture Preparation**

Cultures of myenteric neurons were prepared according to a previously described method (35). A 10-cm segment of ileum was removed, the intestinal contents were flushed, and longitudinal muscle and myenteric plexus were stripped from the intestine. The tissue was digested in Krebs solution containing 1.25 mg/ml collagenase, 1 mg/ml protease, and 0.4% bovine serum albumin. Three centrifugation (350 g) and washing cycles were used to separate the digested tissue under a ×40 binocular dissection microscope and plated in culture dishes with coverslipped bottoms. The culture medium was changed every third day and consisted of Medium 199 enriched with 10% fetal bovine serum and 50 ng/ml nerve growth factor (NGF); the glucose concentration was adjusted to 30 mM. Streptomycin and penicillin were added at 100 µg/ml and 100 U/ml, respectively. The cultures were kept in an incubator at 37°C and continuously gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2}.

**Drugs and Chemicals**

SNX-482, ω-Conotoxin MVIIA, ω-agatoxin IVa, and NGF were obtained from Alomone Laboratories. Ham’s F-12, M-199, and anti-
biotics were purchased from Invitrogen. Fluo-4 AM was obtained from Molecular Probes. CRF human/rat was purchased from Calbiochem. Stresscopin human was obtained from Phoenix Pharmaceuticals. Astressin human/rat was purchased from Bachem-Europe. CP 12,543 was kindly provided by Johnson and Johnson R&D. Nicardipine and TTX were purchased from Sigma/ RBI. All chemicals in the Krebs solutions were purchased from Merck.

### Data Analysis

Silicon Graphics movie files were recorded during confocal scanning. Laser excitation always started 6 s before the beginning of the recording. A control recording with the standard Krebs perfusion was performed during 60 s to check for spontaneous activity. Movies were transported via a file transfer protocol site to a personal computer. Images were then uploaded as a stack in a Tag image file format in Scion image. An in-house developed computer routine (Scion image) was developed to adjust for residual tissue movement. This routine calculated a spatiotemporal map of x-y movement of an intensity spot in the image. The coordinates obtained were subsequently used to reproject the image stack in a larger pixel frame around the stabilized x-y position of the intense spot. A second software routine was developed to calculate fluorescence intensity in different ROIs. Image-intensity histograms were calculated for each region and each image, and a list of average intensities was generated. These averages were subsequently copied to a spreadsheet (Excel) for further analysis.

Relative fluorescence was calculated as \( F_{i} = F_{i, \text{back}}/F_{0} - F_{\text{back}} \), with \( F_{0} \) being the baseline fluorescence at the beginning of the recording and \( F_{i, \text{back}} \) being the fluorescence in a background region. A response to a stimulus was defined as a transient rise in relative fluorescence with an amplitude of at least twice the baseline noise. \( T_{25\text{S}} \) was used as a measure of the duration of the response and was defined as the time between the start of the CRF-induced \( Ca^{2+} \) response and the time at which the signal was down to 25% of its maximum intensity. All results are presented as averages ± SE.

The proportion of responding neurons was expressed relative to total number of neurons identified with a high-K⁺ depolarization. Proportions of neurons responding to different stimuli were statistically analyzed using a Fisher’s exact test. Response characteristics such as amplitude and duration were compared using an unpaired Student’s t-test. \( P \) values of <0.05 were considered statistically significant.

### RESULTS

In total, we identified 1,076 neurons in 46 myenteric ganglia (1 ganglion per tissue) from 16 animals by application of a high-K⁺-Krebs solution. On average, the relative fluorescence rose to 1.73 ± 0.03 (\( n = 214 \) for calculation of response characteristics).

CRF induces \( Ca^{2+} \) transients in myenteric neurons by direct CRF-1 receptor activation. Administration of CRF (10⁻⁷ and 10⁻⁶ M) during 30 s induced a response in 22.4% of the myenteric neurons (\( n = 303, 12 \text{ tissues, 3 animals; Fig. 1} \)). There was no significant difference in response rate to either concentration: 23.8% at 10⁻⁷ M vs. 21.5% at 10⁻⁶ M (\( n = 122 \) and 181, respectively, \( P = 0.67, 95\% \) confidence interval (CI) 0.6–1.4; Fig. 2). On average, relative fluorescence increased to 1.12 ± 0.01, the onset of the response occurred 33.1 ± 3.1 s after the start of CRF application, and the \( T_{25\text{S}} \) was 21 ± 1.7 s (\( n = 66 \) for calculation of response characteristics).

A subset (12/68, 17.6%) of the responding neurons displayed a second \( Ca^{2+} \) transient on activation with CRF. This second response had an amplitude of 1.11 ± 0.02, occurred 81.6 ± 6.4 s after the onset of CRF perfusion, and lasted (\( T_{25\text{S}} \) ± 17 ± 2.9 s.

The CRF-2 agonist stresscopin did not induce any responses at 10⁻⁷ M, whereas a 10-fold higher concentration (10⁻⁶ M) elicited a response in 15.3% of the neurons (\( n = 59, 3 \text{ tissues, 2 animals} \)). However, these responses were significantly blocked (2.7%) in the presence of the specific CRF-1 antagonist CP 154,526 (\( n = 73, 3 \text{ tissues, 2 animals, } P = 0.01, 95\% \) CI 1.2–24.8; Fig. 2).

To test whether secondary neurotransmission might be responsible for some of the CRF-induced responses, we performed similar experiments in the presence of TTX (10⁻⁶ M). After incubation with TTX (\( n = 70, 4 \text{ tissues, 1 animal} \)) for at least 5 min, CRF (10⁻⁶ M) induced a \( Ca^{2+} \) transient in 28.6%, which did not differ statistically from control conditions (\( P = 0.25, 95\% \) CI 0.5–1.2; Fig. 2), and secondary responses were present in 15% of the responding neurons, also not different from control. However, the \( Ca^{2+} \)-transient amplitude was reduced in the presence of TTX (1.08 ± 0.01, \( P < 0.0001, 95\% \) CI 3.7–27.6; Fig. 2).

CRF induced \( Ca^{2+} \) transients require extracellular calcium influx through voltage-sensitive \( Ca^{2+} \) channels (VOCCs). After the normal Krebs solution was replaced with a low-calcium/ high-magnesium Krebs solution, CRF (10⁻⁶ M) could not induce any response (\( n = 47, 2 \text{ tissues, 1 animal; } P < 0.0001, 95\% \) CI –2 to +2; Fig. 2), and secondary responses were present in 15% of the responding neurons, also not different from control. However, the \( Ca^{2+} \)-transient amplitude was reduced in the presence of TTX (1.08 ± 0.01, \( P < 0.0001, 95\% \) CI 3.7–27.6; Fig. 2).

Additional experiments were performed to assess the contribution of different types of VOCCs in the CRF-induced \( Ca^{2+} \) responses in the presence of an L-type \( Ca^{2+} \) channel blocker (Fig. 3).

The N-type \( Ca^{2+} \)-channel blocker \( \omega \)-conotoxin MVIIA (5 × 10⁻⁷ M; \( n = 62, 4 \text{ tissues, 1 animal} \)) or P/Q-type \( Ca^{2+} \)-channel blocker \( \omega \)-agatoxin IVa (5 × 10⁻⁷ M; \( n = 40, 3 \text{ tissues, 1 animal} \)) alone were not able to decrease the number of responding neurons significantly (11.3%, \( P = 0.09, 95\% \) CI 0.9–4.0 and 12.5%, \( P = 0.27, 95\% \) CI 0.72–4.1, respectively).

Simultaneous blocking of N- and P/Q-type VOCCs (\( n = 58, 3 \text{ tissues, 1 animal} \)) reduced the number of CRF-responsive neurons significantly to 8.6% (\( P < 0.05, 95\% \) CI 1.03–6.04). In the presence of the specific R-type \( Ca^{2+} \) blocker SNX-482 (10⁻⁸ M; \( n = 64, 3 \text{ ganglia, 1 animal} \), 10⁻⁶ M CRF-
induced responses were significantly inhibited to 1.5% ($P < 0.0001$, CI 1.9–98.4). Therefore, these data suggest that CRF-induced Ca$^{2+}$ transients are highly dependent on R-type VOCCs. Furthermore, after all these VOCCs were blocked, the responses to CRF were significantly delayed. On average, they started 64.2 s after the onset of the CRF application. ($P < 0.05$ unpaired $t$-test, 95% CI 0.2–41.6).

As described in the MATERIALS AND METHODS, all experiments are performed in the presence of the L-type Ca$^{2+}$ channel blocker nicardipine ($10^{-6}$M). Due to contraction of the underlying longitudinal muscle layer, it is at present not possible to perform Ca$^{2+}$-imaging experiments in tissue preparations in the absence of L-type Ca$^{2+}$ blockade. To check the involvement of L-type Ca$^{2+}$ channels, we chose to use primary cultures of myenteric neurons. CRF ($10^{-7}$ M) was perfused onto Fluo-4 AM-loaded myenteric neurons, and changes in intracellular Ca$^{2+}$ were recorded (41 neurons, 3 culture wells). Eleven neurons displayed transient responses, which started 16.1 ± 2.7 s after the onset of the CRF application (Fig. 4). All responders (25%) displayed a multiple spike pattern, and peak amplitude was determined by averaging two to three peaks. In the presence of nicardipine ($10^{-6}$ M), similar response patterns were observed in the same proportion of neurons.

Fig. 1. Corticotropin-releasing factor (CRF) induces Ca$^{2+}$ transients in myenteric neurons. This figure illustrates the change in relative fluo-4 fluorescence (R.F.) in response to a 30-s application of CRF ($10^{-7}$ M). A: averaged picture of the K$^{+}$-induced Ca$^{2+}$ transient that is illustrated. The ganglion is outlined for reasons of clarity, and 3 neurons, neurons 1–3, are indicated. B–E: images taken from the stack at specific time points (as indicated) and corresponding to the dotted lines in F. F: graph representatively illustrates the changes in R.F. in response to CRF. The 3 tracings correspond with the respective neurons. A control tracing during Krebs solution perfusion precedes the actual stimulation with CRF.

Fig. 2. CRF-induced Ca$^{2+}$ transients are mediated via CRF-1 receptors. This graph displays the number of responding neurons to CRF or stresscopin in different conditions. The neuronal responses are primarily due to CRF-1 receptor activation. The number of responses in the presence of TTX is comparable with control condition, suggesting that they are not synaptically driven. CRF $10^{-6}$ or $10^{-7}$ M, stimulation with corresponding dose of CRF; TTX, stimulation with CRF $10^{-6}$ M in the presence of $10^{-6}$ M TTX; stresscin, stimulation with CRF $10^{-7}$ M in the presence of the nonselective CRF antagonist astressin $10^{-6}$ M; CP 154,526, stimulation with $10^{-6}$ M CRF in the presence of $10^{-7}$ M selective CRF-1 antagonist CP 154,526, CRF receptor antagonists were always used in a 10-fold higher concentration than the agonist and were incubated during at least 5 min before stimulation with the agonist. *$P < 0.0001$ compared with control CRF application. §$P = 0.01$ stresscin vs. stresscin in the presence of CP 154,526.
neurons. The peak amplitudes were also not different in the presence of nicardipine compared with control (1.39 ± 0.03 vs. 1.35 ± 0.04, \( p = 0.46 \)), suggesting no major contribution of L-type Ca\(^{2+}\) current to the intracellular Ca\(^{2+}\) changes.

**Immunohistochemical Staining of CRF-Responsive Neurons and for CRF Receptors**

Single-labeling studies for ChAT and double-labeling studies for calbindin and calretinin were performed. In two ganglia stained for ChAT, we could successfully stain and relocate 14 of 43 neurons. Eight of fourteen ChAT-reactive neurons responded to CRF application. Double labeling for calbindin and calretinin in two ganglia (70 neurons) identified eight calretinin-positive neurons and seven calbindin-positive neurons. Two calretinin-reactive and one calbindin-reactive neurons responded to CRF (see Fig. 5 for illustration).

Immunohistochemical staining for CRF receptors (Fig. 6) showed a differential expression of CRF-1 and -2 receptors at the protein level. The CRF-1 receptor staining clearly labeled individual cell bodies in the ganglia, whereas the CRF-2 receptor staining was markedly more diffuse and barely above background and definitely did not label any individual cell bodies. Double labeling with nitric oxide synthase (NOS) revealed that all (~99%) neurons expressing CRF-1 receptor staining did not express NOS.

**DISCUSSION**

Using confocal calcium imaging, we demonstrated that about one-fifth of myenteric neurons in the guinea pig jejunum display a Ca\(^{2+}\) transient in response to CRF application. Neuronal activation was mainly mediated by CRF-1 receptor activation. We furthermore showed that these responses depend on extracellular Ca\(^{2+}\) and involve several (mainly R type) voltage-operated Ca\(^{2+}\) channels as well as TTX-sensitive sodium channels. Finally, we showed that CRF-1 receptors are expressed in NOS-negative myenteric neurons and that at least subsets of the CRF-responsive neurons display immunoreactivity for calbindin, calretinin, and ChAT.

CRF has attracted increasing interest over the last few years, as different animal studies showed an important effect of CRF on gastrointestinal motility regulation, providing evidence for...
both central and peripheral sites of action. Intracisternally administered CRF inhibited gastric emptying via a mechanism that involved central CRF-2 receptor activation and depended on the vagus nerve (1, 4, 20, 33). However, more recent studies also support a peripheral site of action that might coexist with the centrally mediated effect. Martinez et al. (21) showed that peripherally administered CRF induces a decreased colonic transit time via a CRF-1 receptor-dependent mechanism and a delayed gastric emptying time via CRF-2 receptor activation. Because peripherally administered CRF is not likely to cross the blood-brain barrier (22, 23), these effects seem to be mediated via a direct effect on enteric neurons or smooth muscles in the gastrointestinal tract. Especially the excitatory CRF-1 effect on the colon could not be mimicked or antagonized by intracisternal administration of CRF or specific antagonists (17).

Indirect evidence for an enteric neuronal site of action for CRF was obtained in muscle strip studies showing a TTX-sensitive CRF-induced mechanical and myoelectrical activity in the rat colon (17, 19). Recently, Porcher et al. (28) showed that CRF-1 and CRF-2 receptors were localized in fibers and neurons of myenteric and submucosal ganglia in rat small intestine. Direct evidence for an enteric neuronal effect of CRF is rather limited. Miampamba et al. (24) showed an increase in Fos immunoreactivity after intraperitoneal injection of CRF in ChAT-positive neurons. This action was CRF-1 dependent. Earlier, Hanani and Wood (11) performed electrophysiological studies showing a CRF-induced prolonged depolarization and increased excitability in ~50% of guinea pig ileum myenteric neurons (11). Our data provide further support for a direct effect of CRF on the enteric neurons, because we found a TTX-insensitive excitatory effect in 22% of myenteric neurons. Furthermore, we showed in this study that also in the small intestine, in particular the jejunum, this peripheral neuronal effect is a CRF-1 receptor-mediated mechanism. Although a subset of neurons responded to high concentrations of the specific CRF-2 agonist stresscopin, this was abolished in the presence of selective CRF-1 receptor inhibition by CP 154,526, suggesting that the effect of stresscopin was due to a nonselective action on CRF-1 receptors. This is similar to the recent finding by Liu et al. (15) demonstrating a CRF-1-dependent depolarization of guinea pig myenteric neurons. Moreover, Porcher et al. (28) demonstrated a preferential expression of CRF-1 receptors in the duodenum in the rat, whereas CRF-2 receptors were more expressed in the rat ileum. Our data show that also in the proximal part of the guinea pig small intestine the CRF-1 receptor is functionally expressed. We demonstrated that a subset of the CRF-responsive neurons display immunoreactivity for calbindin, calretinin, and ChAT. This is in line with findings in previous studies. Miampamba et al. (24) showed an increased Fos expression only in ChAT-positive neurons, which represent the excitatory neurons. They did not observe an increase in Fos expression in inhibitory, NADPH-diaphorase-positive neurons. Similarly, we could not find a colocalization between CRF-1 receptor and

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Fig. 5. Immunohistochemical identification of CRF responsive neurons. This figure illustrates the response of 2 calretinin-positive neurons to application of CRF $10^{-7}$ M during 30 s. A: change in R.F. in time after application of $10^{-7}$ M CRF (indicated by the black bar) in 2 calretinin-positive neurons, indicated by the arrows. B: staining for calbindin (in red) and calretinin (in green). C: the corresponding averaged image of the ganglion during high-K Krebs application. The box corresponds to the location of the confocal image (bar = 50 μm).
NOS immunoreactivity. Hanani and Wood (11) described depolarization in Dogiel type 2 neurons, which are known to correlate with calbindin-positive neurons. The latter are believed to be the primary afferent neurons (13). We showed additionally that calretinin-positive neurons can respond to CRF. These neurons have Dogiel type 1 morphology and are either ascending interneurons or motoneurons projecting to the longitudinal muscle (5, 6). Recently Liu et al. (15) also showed that CRF-1 receptor immunoreactivity colocalizes with calbindin, ChAT, and substance P. These immunohistochemical data correlate well with the general excitatory effect of CRF-1 receptor activation on intestinal motility.

Hanani and Wood (11) showed that CRF-induced depolarization was cAMP mediated and involved closure of KV110 channels. One of the signaling pathways following CRF receptor activation is indeed a Gs protein-coupled process causing an increase in adenylate cyclase (AC) activity and subsequent increase of intracellular cAMP and PKA activity (9, 10). The fact that Ca^{2+} transients were abolished after removal of extracellular Ca^{2+} is compatible with the hypothesis that CRF signaling in myenteric neurons involves primarily AC-dependent pathways. Primary activation of the phosphatidylinositol-2′-phosphate pathway would have occurred independently of changes in extracellular Ca^{2+}.

We provided in our study evidence that the major contributors to the observed Ca^{2+} transients are indeed R-type VOCCs and, to a lesser extent, P/Q- and N-type VOCCs. L-type channels do not significantly contribute to the CRF-induced responses as shown by experiments in cultured myenteric neurons. T-type VOCCs have not clearly been identified in myenteric neurons (31).

Inhibition of action potential firing by TTX decreased the amplitude of the Ca^{2+} transients significantly. This indicates that a proportion of the transients was most likely generated by additional recruitment and opening of VOCCs by action potential firing. Even after blocking L-, P/Q- and N-type VOCCs, we still observed CRF-induced Ca^{2+} responses. The exact role of P/Q- and N-type channels in CRF-induced Ca^{2+} signaling needs be further clarified in future studies. Simultaneous blocking of the channels significantly reduces the CRF response.
although separate inhibition of either channel does not cause a statistically significant inhibition, which may be due to the small number of ganglia studied in these specific conditions. However, blocking R-type channels caused a nearly complete inhibition of the CRF-induced Ca\(^{2+}\) transients. This gives further support to the recently reported role of R-type VOCCs in somatic Ca\(^{2+}\) influx in myenteric neurons by Bian et al. (2).

The fact that the number of responding neurons in our study is lower compared with electrophysiological studies (11) can be explained by the difference in technique. First, using confocal calcium imaging, we study all neurons in a ganglion. In contrast, electrophysiological studies are limited to impalement of individual neurons. Electrophysiological studies are less likely to sample all neurons, because it is influenced by the size of their soma and their location within the ganglion. On the other hand, some underestimation of responses might occur with our technique due to the lower signal-to-noise ratio. It is conceivable, for instance, that due to a limited expression of their soma and their location within the ganglion. Czimmer J, Million M, and Tache Y.

**REFERENCES**