Characterizing voltage-dependent Ca\(^{2+}\) channels coupled to VIP release and NO synthesis in enteric synaptosomes

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Kurjak, M., A. Sennefelder, M. Aigner, V. Schusdziarra, and H. D. Allescher. Characterizing voltage-dependent Ca\(^{2+}\) channels coupled to VIP release and NO synthesis in enteric synaptosomes. Am J Physiol Gastrointest Liver Physiol 283: G1027–G1034, 2002. First published July 31, 2002; 10.1152/ajpgi.00400.2001.—In enteric synaptosomes of the rat, the role of voltage-dependent Ca\(^{2+}\) channels in K\(^+\)-induced VIP release and nitric oxide (NO) synthesis was investigated. Basal VIP release was 39 ± 4 pg/mg, and cofactor-substituted NO synthase activity was 7.0 ± 0.8 fmol·mg\(^{-1}\)·min\(^{-1}\). K\(^+\) depolarization (65 mM) stimulated VIP release Ca\(^{2+}\) dependently (basal, 100%; K\(^+\), 172.2 ± 16.2%; P < 0.05, n = 5). K\(^+\)-stimulated VIP release was reduced by blockers of the P-type (ω-agatoxin-IVA, 3 × 10\(^{-8}\) M) and N-type (ω-conotoxin-GVIA, 10\(^{-6}\) M) Ca\(^{2+}\) channels by ∼50 and 25%, respectively, but not by blockers of the L-type (isradipine, 10\(^{-8}\) M), Q-type (ω-conotoxin-MVIC, 10\(^{-6}\) M), or T-type (Ni\(^{2+}\), 10\(^{-6}\) M) Ca\(^{2+}\) channels. In contrast, NO synthesis was suppressed by ω-agatoxin-IVA, ω-conotoxin-GVIA, and isradipine at ∼79, 70, and 70%, respectively, whereas Ni\(^{2+}\) and ω-conotoxin-MVIC had no effect. These findings are suggestive of a coupling of depolarization-induced VIP release primarily to the P- and N-type Ca\(^{2+}\) channels, whereas NO synthesis is presumably dependent on Ca\(^{2+}\) influx not only via the P- and N- but also via the L-type Ca\(^{2+}\) channel. In contrast, none of the Ca\(^{2+}\) channel blockers affected VIP release evoked by exogenous NO, suggesting that NO induces VIP secretion by a different mechanism, presumably involving intracellular Ca\(^{2+}\) stores. synaptosomes; enteric nervous system; vasoactive intestinal polypeptide; voltage-dependent Ca\(^{2+}\) channels; nitric oxide synthase

The activation of voltage-dependent Ca\(^{2+}\) channels (VDCCs) is a key regulatory step in the process of excitation-secretion coupling (4, 25). Molecular neurobiology has revealed a diversity of Ca\(^{2+}\) channel subtypes (for review, see Refs. 15 and 22). They are classified according to their α\(_1\)-subunit, which is encoded by seven different genes, and subdivided into classes A (P/Q-type), B (N-type), C and D (L-type), S (L-type), G (T-type) and E (R-type) Ca\(^{2+}\) channels (15). Cumulative evidence has been presented that multiple VDCCs may coexist in a single synapse to regulate neurosecretion from peripheral autonomic (31) and enteric nerves (37, 41, 43). Immunohistochemistry revealed α\(_1\)B and α\(_1\)A channel-like immunoreactivity in nerve processes within the myenteric plexus of the rat small intestine, suggesting the presence of N- and P-type Ca\(^{2+}\) channels, respectively (26). This pattern of distribution closely resembles the localization of both channels in the central nervous system (34, 38). An α\(_1\)C immunoreactivity, implying the presence of L-type Ca\(^{2+}\) channels, appeared to be more confined to ganglionic structures than to nerve processes (26). Furthermore, binding sites for both ω-conotoxin-GVIA (ω-Ctx GVIA), a blocker of the N-type Ca\(^{2+}\) channel, and nitrendipine, a blocker of the L-type channel, had been demonstrated on isolated synaptosomes of the canine small intestine (1), suggesting a role of both channel types in neurosecretion. However, the functional relevance of these findings remains to be elucidated.

VIP represents an inhibitory nonadrenergic, noncholinergic (NANC) neurotransmitter in the gastrointestinal tract that acts in concert with nitric oxide (NO) to relax smooth muscle. Both VIP and the NO-synthesizing enzyme neuronal NO synthase (nNOS) are colocalized in a subset of descending inhibitory neurons in the rat (2, 17). Previously, it has been shown that enteric synaptosomes release VIP in response to exogenous NO and in a Ca\(^{2+}\)-dependent manner following stimulation with KCl (3) and that a constitutive Ca\(^{2+}\)-dependent NO activity is retained within isolated nerve terminals from rat small intestine (23), which is functionally coupled to VIP release (29). VIP output from longitudinal muscle preparations with adherent myenteric plexus (LM/MP) has been shown to be reduced by ω-Ctx GVIA, a blocker of the N-type Ca\(^{2+}\) channel (11, 13). Since NO is known to relax smooth muscle directly or indirectly by releasing VIP (3, 21), and since the effect of NO on smooth muscle is antagonized in the presence of ω-Ctx GVIA (6, 11, 13), it was of interest to investigate the effect VDCC blockers on NO-induced VIP release as well. Furthermore, it must be clarified whether selective blockade of VDCCs other than the N-type channel elicits an effect on VIP release or NO synthesis.

On the ultrastructural level, NOS appears not to be located in the same domain of Ca\(^{2+}\) entry as the VIP-
containing vesicles (5), leaving the possibility that both transmitters are controlled independently. So far, no data have been presented regarding the involvement of distinct Ca\(^{2+}\) channels in both the release of VIP from enteric nerve terminals by studying Ca\(^{2+}\) blockers targeting subsets of N-type (\(\omega\)-Ctx GVIA), L-type (isradipine), T-type (\(\mathrm{Ni}^{2+}\)), and P/Q-type [\(\omega\)-agonistxin-IVA] and \(\omega\)-conotoxin-MVIIC [\(\omega\)-Ctx MVIIC] Ca\(^{2+}\) channels and 2) to study the effect of the respective Ca\(^{2+}\) channel blockers on NO production by nNOS. These questions have been addressed (28) by studying isolated nerve terminals that offer the unique opportunity to examine intracellular and subcellular mechanisms of neurotransmitter release without the interference of other local or systemic factors present in vivo or in the intact organ in vitro.

**MATERIALS AND METHODS**

**Preparative Techniques**

**Tissue handling and membrane preparation.** Synaptosomes were prepared as described previously (28). Briefly, five male Wistar rats were killed by cervical dislocation, and the small intestine was quickly removed and suspended in ice-cold buffer (20 mM MOPS, 10 mM MgCl\(_2\), 8% wt/vol sucrose, pH 7.4). All further preparative steps were carried out at 0-4°C. Approximately 6- to 8-cm pieces of small intestine were dissected, cleaned of mesenteric arcade and fat, and opened along the mesenteric attachment line. The mucosal layer was scraped off with a sharp razor blade, and the remaining muscle layers were put into cold buffer. The muscle tissue was blotted dry on filter paper and weighed. For membrane preparation, the tissue was resuspended in isolation buffer (8% wt/vol sucrose, 20 mM MOPS, pH 7.4), minced with scissors, and homogenized with a Polytron PT20 homogenizer at -1,500 rpm setting for 15 s (5 × 5 s).

Fractionation of tissue homogenate by differential centrifugation. The tissue homogenate was centrifuged in two steps of 800 g for 10 min to remove myofibrils and remaining nuclei. The supernatant was collected [postnuclear supernatant (PNS)] and centrifuged at 3,500 g for 10 min to obtain the P1 fraction. The supernatant was centrifuged again at 100,000 g for 90 min. The pellet from this centrifugation (microsomal 1 (MIC1)) was resuspended and centrifuged again at 10,000 g for 10 min. The resulting pellet and the supernatant are referred to as mitochondrial 2 (P2) and microsomal 2 (MIC2) fractions, respectively.

Differential centrifugation led to a substantial enrichment of L-[\(3\)H]arginine in the fraction P2 (8-fold (44.9 ± 8 fmol/mg protein) vs. PNS (5.5 ± 1.7 fmol/mg protein)) and was paralleled by a 7.2-fold increase in the content of VIP in the P2 fraction (6.4 ± 1.9 vs. 0.9 ± 0.3 pmol/mg protein in PNS), as reported previously (3). With respect to the methodical approach employed, the synaptosomal fraction consists of a mixed population of nerve terminals, including nerve endings of motoneurons, from different animals.

**Analytic Techniques**

**Protein assay.** Protein was measured spectrophotometrically according to the method of Bradford (8). Bovine serum \(\gamma\)-globulin was used as standard.

Radioimmunoassay. VIP immunoreactivity was determined as described elsewhere (35). The porcine VIP antibody (provided by S. R. Bloom, Royal Postgraduate Medical School, London, UK) showed no interaction with NH\(_2\)-terminal fragments of VIP, secretin, peptide histidine isoleucine, growth hormone-releasing factor, gastric inhibitory peptide, or pituitary adenylate cyclase-activating peptide (personal communication, S. R. Bloom). 

Peptide release. Peptide release studies were carried out in Krebs-Ringer-bicarbonate solution (KRS; in mM: 115.5 NaCl, 1.16 MgSO\(_4\), 1.16 NaH\(_2\)PO\(_4\), 11.1 glucose, 21.9 NaHCO\(_3\), 2.5 CaCl\(_2\), 4.16 KCl) gassed with 95% O\(_2\)-5% CO\(_2\). In experiments designed to study the role of Ca\(^{2+}\)-free medium, CaCl\(_2\) was omitted and 0.5 mM EGTA was added. KRS (1,050 μl) and 150 μl of drugs or KRS alone serving as a control (basal level) were incubated in separate test tubes at 37°C in a gently shaking water bath. The reaction was started by adding 300 μl of synaptosomal membranes (300 μg protein) to each tube at timed intervals. To study the effect of Ca\(^{2+}\) channel blockade, the blockers were incubated with the synaptosomes for 0, 5, 15, and 20 min. According to the data obtained from the time-course experiments, the incubation for all subsequent experiments lasted 15 min. To stop the reaction, the synaptosomal membranes were put on ice and immediately sedimented by high-speed centrifugation in a refrigerated centrifuge. The supernatant was withdrawn and immediately frozen at -20°C until peptide determination by radioimmunoassay.

**Assay of NOS activity.** NOS activity was determined by monitoring the formation of L-citrulline from L-arginine by a modification of methods described previously (9). Enzymatic reactions were conducted at 37°C in 25 mM MOPS-8% sucrose buffer containing 1 mM NADPH, 0.1 μM tetrahydrobiopterin, 1 μM calmodulin, 1 mM CaCl\(_2\), 0.1 μM FAD, 0.1 μM flavin mononucleotide (FMN), and other test agents as specified later, in a final incubation volume of 750 μl. The L-[\(3\)H]arginine was purified by anionic exchange chromatography on columns of Dowex 1×8, OH-form to remove traces of L-[\(3\)H]citrulline. After preincubation for 30 min at 4°C with synaptosomes (500 μl), the enzymatic reactions were started by adding ~500,000 dpm of L-[\(3\)H]arginine (63 Ci/mmol) and terminated after 15 min by immediate heating to 90°C for 6 min and addition of 1 ml distilled water containing 1 mM L-arginine and 1 mM L-citrulline. Samples were applied to columns containing 1 ml of Dowex AG 50 W X 8 resin, Na\(^+\) form, preequilibrated with sucrose MOPS buffer. The eluate (2 ml) was collected in a liquid scintillation vial. After the addition of 1.5 ml scintillation fluid, samples were counted in a Beckman LS 3801 spectrometer. The recovery of L-[\(3\)H]citrulline in the first 4 ml of the eluate was ~92%; contamination with L-[\(3\)H]arginine did not exceed 2%. Basal values were obtained by heating samples to 100°C for 5 min before incubation. Cofactor-stimulated NOS activity over basal was considered as control. To study the effect of Ca\(^{2+}\)-free medium, CaCl\(_2\) was omitted and 0.5 mM EGTA was added.

**Drugs**

The labeled L-[\(3\)H]arginine (63 Ci/mmol) was purchased from Amersham. All other reagents were purchased from the indicated sources as follows: NADPH, FAD, FMN, citrulline, calmodulin, pepstatin A, dithiothreitol, trypsin inhibitor, Ni-sulfate, glutamate, carbachol, atropine, [\(\alpha\)-penicillamine-

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(2,5)enkephalin (DPDPE), substance P, and naloxone hydrochloride dihydrazine were from Sigma; tetrahydrodipropionin was from ICN Biomedicals (Eschwege, Germany); S-nitroso-
N-acetylpenicillamine (SNAP) and N-methyl-D-aspartate (NMDA) were from Calbiochem (Bad Soden, Germany); thiophen was from Fluka (Munich, Germany); R-4-carboxy-
3-hydroxyphenylglycine, 1-aminoacycloextranecarboxylic acid (ACBC), and endomorphin-1 were from Toeris-Cookson Bio-
trend Chemikalien (Cologne, Germany); [n-Lys(nicotinoyl)1, β-(3-pyridyl)-Ala3,3,4-dichloro-D-Phe5-Asn6-D-Trp7,9,Nle11]-
substance P (Spantide II) was from Bachem (Heidelberg,
Germany); U-50488 was from Upjohn (Kalamazoo, MI); BAY K 8644 and diethylamine/NO complex sodium (DEA-NO) was
from Research Biochemicals International (Natick, MA);
PMSP was from Serva (Heidelberg, Germany); α-Agatx IVA
was from Alexis (Grünberg, Germany); ω-Ctx GVIA and MVIIC were from Alomone Labs (Jerusalem, Israel); and
isradipine was generously provided by Prof. F. Hofmann,
(Dept. of Pharmacology, Technical University of Munich). All
experiments with isradipine were carried out under light
protection. Adequate controls were performed with the vehi-
cles used for solubilizing each reagent.

Statistics

Data are given as means ± SE; n indicates the number of
independent observations in separate experiments from sep-
parate preparations. For each value of a given drug of a single
preparation, the release study was carried out in duplicate.
The values of peptide release experiments showed some vari-
atiation in separate experiments and were therefore expressed
as the relative increase over basal levels (=100%). Analysis
of variance, followed by Dunnett’s post hoc test for multiple
testings, was used to determine statistical significance. For
comparisons of two means, paired or unpaired t-test was
performed. Values of P = 0.05 were considered significant.

RESULTS

Assessment of VIP Release From
Isolated Synaptosomes

Synaptosomes of rat small intestine are capable of
releasing VIP in response to K+ depolarization. Depo-
larization-induced VIP release occurred in a Ca2+-
dependent manner (Fig. 1). Basal release was also
significantly reduced (~40%) when incubation was car-
ried out in a Ca2+-free medium containing 0.5 mM
EGTA. No significant difference was noted between
basal and K+ induced VIP release in the presence of
Ca2+-free medium, suggesting that Ca2+-independent
release is not likely to occur. A first set of experiments
was conducted to investigate the effect of the respective
selective Ca2+ channel blockers on basal VIP release.
The inhibitors were used at concentrations selective for
N-type (ω-Ctx GVIA, 10−6 M), N/Q-type (ω-Ctx MVIIC,
10−6 M), P-type (ω-Agatx IVA, 3 × 10−8 M), L-type
(isradipine, 10−8 M), and R/T-type (Ni2+, 10−6 M) Ca2+
channels. Of the blockers tested, only ω-Agatx IVA
significantly inhibited basal VIP release to an extent
not significantly different from Ca2+-free medium
[basal, 36.8 ± 3.9 pg/mg (100%); ω-Agatx IVA, 86.5 ±
8.8%; P < 0.05; n = 6; Fig. 2]. None of the other
blockers affected basal VIP release (basal, 100%; ω-Ctx
GVIA, 122.1 ± 12.1%; ω-Ctx MVIIC, 140.6 ± 23.6%;
isradipine, 113.8 ± 10.9%; Ni2+, 92.1 ± 16.4%).
VIP release evoked by K⁺. The combination of both blockers resulted in a further inhibition (10⁻⁶ M ω-Ctx GVIA + 3 × 10⁻⁶ M ω-Agatx IVA, 119.6 ± 15.7%; n = 5), which was not significantly different from that of ω-Agatx IVA alone.

The Q-type channel blocker ω-Ctx MVIC had no significant effect [basal, 183.1 ± 3.3 pg/mg (100%); K⁺, 168.6 ± 12.2%; K⁺ + 10⁻⁶ M ω-Ctx MVIC, 163.8 ± 17.5%; n = 5]. The L-type Ca²⁺ channel blocker isradipine (10⁻⁶M) did not suppress K⁺-evoked VIP release, nor did it augment the inhibition in the presence of ω-Ctx GVIA and ω-Agatx IVA [basal, 183.1 ± 3.3 pg/mg (100%); K⁺, 168.6 ± 7.2%; K⁺ + isradipine, 141.3 ± 9.7%; K⁺ + ω-Ctx GVIA + ω-Agatx IVA, 119.6 ± 15.7%; K⁺ + ω-Ctx GVIA + ω-Agatx IVA + isradipine, 136.4 ± 21.5%; n = 5], suggesting that blockade of the L-type Ca²⁺ channel had no significant effect.

BAY K 8644 (10⁻⁶ M), which selectively activates L-type channels, failed to significantly stimulate either basal or K⁺-induced VIP release, respectively [basal, 149.8 ± 32.9 pg/mg (100%); BAY K 8644, 98.6 ± 20.8%; K⁺, 166.7 ± 18.4%; K⁺ + BAY K 8644, 134.8 ± 24.9%; n = 4].

Low-threshold T-type Ca²⁺ channels and high-threshold R-type Ca²⁺ channels are sensitive to blockade by Ni²⁺. Addition of Ni²⁺ (10⁻⁶–10⁻⁴ M) had no significant effect on synaptosomal VIP release evoked by K⁺ [basal, 217.1 ± 3.3 pg/mg (100%); K⁺, 134.0 ± 4.3%; 10⁻⁶ M Ni²⁺, 125.3 ± 26.6%; 10⁻⁵ M Ni²⁺, 159.1 ± 34.9%; 10⁻⁴ M Ni²⁺, 141.9 ± 30.9%; n = 3].

Since the synaptosomal fraction consists of a mixed population of enteric nerves, which could release their neurotransmitter in response to K⁺ depolarization, the observed effect of the VDCCs could be due to an indirect action on other nerve terminals than the VIPergic one. To clarify this, we investigated whether carbachol, substance P, or the opioid peptides had a stimulatory effect on VIP release from nerve terminals. The respective data are shown in Table 1.

Table 1. Effect of the transmitter substances carbachol, substance P, and the agonists at the µ-, δ-, and κ-opioid receptor, endomorphin-1, DPDPE, and U-50488, respectively, on VIP release from enteric synaptosomes

<table>
<thead>
<tr>
<th>Substance</th>
<th>VIP Release, % Basal</th>
<th>Absolute Value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>237.3 ± 19.0 pg/mg</td>
<td>4</td>
</tr>
<tr>
<td>Carbachol, 10⁻⁶ M</td>
<td>116.3 ± 11.4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Carbachol, 10⁻⁵ M</td>
<td>119.9 ± 16.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Carbachol, 10⁻⁴ M</td>
<td>117.3 ± 16.2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Atropine, 10⁻⁶ M</td>
<td>96.0 ± 24.0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>198.9 ± 15.6 pg/mg</td>
<td>5</td>
</tr>
<tr>
<td>Substance P, 10⁻⁷ M</td>
<td>99.1 ± 5.7</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Substance P, 10⁻⁶ M</td>
<td>113.2 ± 8.6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Substance P, 10⁻⁵ M</td>
<td>109.1 ± 13.1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Spantide II, 10⁻⁶ M</td>
<td>147.0 ± 14.4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>135.4 ± 15.1 pg/mg</td>
<td>8</td>
</tr>
<tr>
<td>Endomorphin, 10⁻⁴ M</td>
<td>110.8 ± 18.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>DPDPE, 10⁻⁴ M</td>
<td>110.5 ± 22.5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>U-50488, 10⁻⁶ M</td>
<td>78.8 ± 7.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Naloxone, 10⁻⁷ M</td>
<td>107.7 ± 7.8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. The κ-opioid receptor agonist U-50488 significantly inhibited VIP release. In the presence of the the substance P receptor antagonist Spantide II, VIP release was significantly enhanced, suggesting an inhibitory effect of tonally released substance P. In contrast, the cholinergic muscarinic receptor antagonist atropine and the opiate receptor antagonist naloxone did not influence basal VIP release. DPDPE, 9-3-penicillamine (2, 5) enkephalin. Experiments were performed in duplicate. *P < 0.05.
Effect of Subtype-Specific Ca\(^{2+}\) Channel Blockers on NOS Activity in Enteric Synaptosomes

NOS activity (in fmol·mg\(^{-1}\)·min\(^{-1}\)) was decreased by 61% in the absence of Ca\(^{2+}\) [control (all cofactors included over basal), 7.0 ± 0.8; Ca\(^{2+}\)-free, 2.8 ± 1.4; \(P < 0.05; n = 6\)]. Either treatment with ω-Agatx IVA, ω-Ctx GVIA, or isradipine reduced NOS activity compared with control (control, 7.0 ± 0.8; ω-Agatx IVA, 1.5 ± 1.2; ω-Ctx GVIA, 2.1 ± 1.3; isradipine, 2.1 ± 1.2; either treatment \(P < 0.05; n = 5\); Fig. 5). Each of the three substances significantly reduced prestimulated NOS activity, corresponding to an inhibition by 79, 70, and 70%, respectively. No significant difference was obtained when these treatments were compared with Ca\(^{2+}\)-free medium. In contrast, ω-Ctx MVIC or Ni\(^{2+}\) (control, 7.0 ± 0.8; 10 \(^{-6}\) M ω-Ctx MVIC, 3.4 ± 1.8; 10 \(^{-6}\) M Ni\(^{2+}\), 4.4 ± 1.9; \(n = 5\)) failed to significantly reduce cofactor-stimulated NOS activity (Fig. 5). Higher concentrations of Ni\(^{2+}\) were also not effective (control, 3.1 ± 1.3; 10 \(^{-6}\) M Ni\(^{2+}\), 3.7 ± 1.6; 10 \(^{-5}\) M Ni\(^{2+}\), 3.6 ± 1.7; 10 \(^{-4}\) M Ni\(^{2+}\), 3.1 ± 1.2; \(n = 4\)).

These data suggest that, with respect to NO synthesis, not only P- and N-type channels but also L-type channels are involved, whereas Q- and T-type channels are presumably of minor relevance. Since in the central nervous system NMDA receptor activation induces Ca\(^{2+}\) influx, thereby stimulating NOS, the effect of both NMDA receptor stimulation and blockade was studied. Addition of NMDA or glutamate does not alter cofactor-stimulated NOS activity (control, 2.2 ± 0.1; 10 \(^{-7}\) M NMDA, 2.0 ± 0.3; 10 \(^{-6}\) M NMDA, 1.9 ± 0.3; 10 \(^{-5}\) M NMDA, 1.8 ± 0.4; 10 \(^{-7}\) M glutamate, 2.1 ± 0.3; 10 \(^{-6}\) M glutamate, 2.0 ± 0.3; 10 \(^{-5}\) M glutamate, 1.9 ± 0.2; no treatment was significant vs. control; \(n = 5\)). The NMDA receptor antagonist R-4-carboxy-3-hydroxyphenylglycine also did not modify NOS activity (control, 2.2 ± 0.1; 10 \(^{-6}\) M R-4-carboxy-3-hydroxyphenylglycine, 2.0 ± 0.3; not significant vs. control; \(n = 4\)). Similar results were obtained with ACBC, an NMDA receptor antagonist at the glycine site (control, 2.2 ± 0.1; 10 \(^{-6}\) M ACBC, 2.9 ± 1.1; not significant vs. control; \(n = 4\)).

Since addition of Ca\(^{2+}\) evokes the release of other neurotransmitters, which in turn might stimulate NO production in synaptosomes, a new series of experiments was conducted. The data obtained with carbachol, substance P, and the respective agonists at the opioid \(\mu\), \(\delta\), and \(\kappa\)-receptors, endomorphin-1, DPDPE, and U-50488 are shown in Table 2.

Table 2. Effect of carbachol, substance P, and the agonists at the \(\mu\), \(\delta\), and \(\kappa\)-opioid receptor, endomorphin-1, DPDPE, and U-50488, respectively, on NO synthesis in enteric synaptosomes

<table>
<thead>
<tr>
<th>Substance</th>
<th>NOS activity in fmol·mg(^{-1})·min(^{-1})</th>
<th>(n)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10.7 ± 2.9</td>
<td>4</td>
</tr>
<tr>
<td>Carbachol, 10 (^{-7}) M</td>
<td>10.2 ± 1.5</td>
<td>4</td>
</tr>
<tr>
<td>Carbachol, 10 (^{-6}) M</td>
<td>10.6 ± 1.4</td>
<td>4</td>
</tr>
<tr>
<td>Carbachol, 10 (^{-5}) M</td>
<td>8.1 ± 1.8</td>
<td>4</td>
</tr>
<tr>
<td>Atropine, 10 (^{-6}) M</td>
<td>5.5 ± 2.0</td>
<td>3</td>
</tr>
<tr>
<td>Substance P, 10 (^{-7}) M</td>
<td>2.2 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>Substance P, 10 (^{-6}) M</td>
<td>2.6 ± 1.2</td>
<td>4</td>
</tr>
<tr>
<td>Substance P, 10 (^{-5}) M</td>
<td>2.3 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>Spantide II 10 (^{-6}) M</td>
<td>2.1 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>8.0 ± 2.2</td>
<td>4</td>
</tr>
<tr>
<td>Endomorphin-1, 10 (^{-6}) M ((\mu)-agonist)</td>
<td>7.1 ± 2.8</td>
<td>4</td>
</tr>
<tr>
<td>DPDPE, 10 (^{-6}) M ((\delta)-agonist)</td>
<td>6.1 ± 1.7</td>
<td>4</td>
</tr>
<tr>
<td>U-50488, 10 (^{-6}) M ((\kappa)-agonist)</td>
<td>6.0 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>Naloxone, 10 (^{-6}) M</td>
<td>7.7 ± 0.8</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are means ± SE. The cholinergic muscarinic receptor antagonist atropine, the substance P receptor antagonist spantide II, or the opiate antagonist naloxone did not influence basal nitric oxide (NO) synthesis, indicating that a tonic release of acetylcholine substance P, or opioid peptides does not have any impact on NO production. Experiments were performed in duplicate. No statistically significant effects were found.
Ca²⁺ CHANNELS IN VIP RELEASE

NANC relaxation of gastrointestinal muscle is mediated by NO and VIP, both of which are localized in descending inhibitory interneurons of the enteric nervous system. With respect to smooth muscle relaxation, interaction between these two transmitters appears to be likely; however, it is hard to assess since the enteric nervous system is a complex system and interference of local or systemic factors cannot be ruled out in vivo or in the intact organ in vitro. As an alternative approach, we used a synaptosomal preparation to inquire about the subcellular mechanisms involved in the release of VIP and NO synthesis. With respect to VIP release, previous data from enteric nerve terminals (3) and from isolated ganglia (21) can be confirmed showing that K⁺ is capable of stimulating VIP release Ca²⁺ dependently. NO synthesis also strictly requires Ca²⁺ (23). Additionally, it can be shown that within isolated enteric nerve terminals NO synthesis is both coupled to VIP release (29) and under feedback control of endogenous or exogenous NO (30). There is evidence that NO-mediated relaxation of intestinal smooth muscle is strongly dependent on Ca²⁺ entry through N-type Ca²⁺ channels, which are blocked specifically by ω-Ctx GVIA (7, 11, 13). Accordingly, experiments were performed to investigate the role of different VDCCs in the release of VIP and NO synthesis in enteric synaptosomes. Immunohistochemistry revealed the presence of the α₁A- and α₁B-subunits corresponding to the P- and N-type Ca²⁺ channels as well as the α₁C- and α₁D-subunits corresponding to the L-type Ca²⁺ channel in the enteric nervous system, respectively (26). The data presented here clearly indicate that, following K⁺ depolarization, N- and P-type Ca²⁺ channels are apparently coupled to VIP release, which extends previous findings in LM/MP preparations (13). Basal VIP output presumably is under control of the P-type Ca²⁺ channel. However, the synaptosomal fraction contains a mixed population of nerve endings, releasing their respective neurotransmitters in the presence of depolarizing stimuli. Assuming that some of these nerve endings synthesize a transmitter that stimulates VIP release, it could be speculated that the VDCCs might act indirectly and not at VIPergic nerve terminals. In another series of experiments, we inquired about a possible effect of other transmitters on VIP release. The “chemical coding” of the rat gut is quite different from other species (18). Inhibitory motor neurons contain almost exclusively NOS and/or VIP. The transmitters of excitatory motor neurons are acetylcholine, substance P, and/or opioid peptides, whereas the secretomotor neurons contain either VIP or substance P (18). Other peptidergic transmitters, detected by immunohistochemistry in the rat enteric nervous system, like somatostatin, neuromedin U, neurotensin, and CGRP, are almost exclusively confined to enteric ganglia. Within nerve fibers supplying the smooth muscle, they are observed in small numbers only (16). Enteric ganglia, however, are separated by the means of differential centrifugation during the preparation of synaptosomes (28). With respect to recently published functional data showing that both galanin and neuropeptide Y, which are present in moderate numbers within nerve fibers to smooth muscle (16), inhibit VIP output from the LM/MP preparation (12), the transmitters substance P, acetylcholine, and opioid peptides appeared to be the most likely candidates to stimulate VIP release. Our data show that none of the latter transmitters has a stimulatory effect on VIP release, suggesting that the VDCCs are acting directly on VIPergic nerves.

A second series of experiments was conducted to investigate the role of Ca²⁺ channels in NO production. Our data confirmed and extended previous findings (13) showing that not only N-type but also L-type and P-type Ca²⁺ channels provide Ca²⁺ influx into the nerve terminal to enable NO production. In analogy to the VIP assay, we investigated whether the effect is due to an indirect modulatory effect of VDCCs on nerve terminals containing either substance P, acetylcholine, or opioid peptides, which in turn could stimulate NO production. The latter substances did not modify NOS activity, suggesting a direct modulatory effect of the VDCCs on NOS-containing neurons.

Interestingly, NO biosynthesis in the brain is regulated by NMDA receptor-activated Ca²⁺ influx (19, 20). In enteric synaptosomes, both NMDA/glutamate and the NMDA receptor antagonists failed to influence NO synthesis significantly. These results indicate that distinct Ca²⁺ influx pathways specifically regulate nNOS activity in various tissues.

Further experiments were conducted to clarify the role of VDCCs in NO-mediated VIP release. In contrast to depolarization-induced VIP release, the NO-induced VIP secretion was not blocked in the presence of Ca²⁺ channel blockers. There is cumulative evidence that NO triggers exocytosis by bypassing requirements of Ca²⁺ influx (33, 40). NO could accordingly release VIP in different ways, interacting with the release machinery (32), modulating ion channels on the membrane (27), or activating intracellular signal transduction systems (42). The last of these has been demonstrated for NO-induced VIP release from enteric synaptosomes, since it could be antagonized in the presence of blockers of the cGMP/protein kinase G pathway (29).
Previous studies in the central nervous system have shown that cGMP analogs and NO donors reduce symptomatic [Ca^{2+}]{(33)}, which appears to be incompatible with the generally accepted Ca^{2+}-dependent requirement for exocytosis. Our data support the notion that VIP release, although normally highly Ca^{2+}-dependent, might occur in a Ca^{2+}-independent manner. Further experiments are needed to clarify the exact mechanism involved.

From the data presented, clear evidence could be obtained that the suppression of NO-mediated intestinal relaxation by the N-type Ca^{2+} channel antagonist ω-Ctx GVIA (6, 13) is presumably not due to inhibition of NO-induced VIP release. The data also show that NO production and VIP release by depolarizing stimuli are differentially regulated with respect to the involvement of L-type channels. The functional role of these L-type channels in NO synthesis needs to be clarified. The L-type channel is encoded by the α_{1C} and α_{1D}-subunits. The presence of both α_{1C} and α_{1D}-subunits in enteric nerve terminals had been demonstrated by immunocytochemistry (26). L-type channels apparently are involved in neurotransmitter release from cerebellar synapses (36), NANC nerves supplying the iris muscle (24), and dendrites of hippocampal granule cells (39), but its involvement in the release process of neuropeptides in the enteric nervous system has not yet been established. Another concept for the functional role of L-type channels in neuronal tissue is provided by studies in hippocampal neurons. It could be shown that mobilization of calmodulin from cytosolic sources can be induced by certain Ca^{2+} entry systems, like L-type channels or NMDA receptors, but not by N- or P-type channels (14). Since binding of calmodulin to NOS is prerequisite for NO production and nNOS itself is targeted to specific intraterminal regions (10), the mobilization of calmodulin could be functionally related to the L-type channel. However, this is speculative and deserves further elucidation.

In conclusion, we demonstrated that VIP release following K+ depolarization is coupled to N- and P-type Ca^{2+} channels, whereas NO synthesis is presumably dependent on Ca^{2+} influx via the N-, P-, and L-type Ca^{2+} channels. However, VDCCs appear not to be functionally coupled to the VIP release in response to extracellular NO, implying the possibility that intracellular Ca^{2+} stores, for example of mitochondrial origin, are of central importance for an interplay between the two messenger substances NO and Ca^{2+}.

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REFERENCES


