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Calcium Uptake and Protein Phosphorylation in Myenteric Neurons, Like the Release of Vasoactive Intestinal Polypeptide and Acetylcholine, Are Frequency Dependent

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Abstract: The mechanism of the electrical-to-chemical decoding involved in the preferential release of the transmitters acetylcholine and vasoactive intestinal polypeptide (VIP) by electrical field stimulation at low (5 Hz) and high (50 Hz) frequencies was studied in superfused myenteric neurons. The stimulation-induced uptake of $^{45}Ca^{2+}$ accompanying high frequency stimulation was markedly reduced by 10 μM nifedipine, a specific blocker of L-type voltage-sensitive Ca^{2+} channels (VSCCs), as was also the preferential high-frequency release of VIP. By contrast, the $^{45}Ca^{2+}$ uptake during low-frequency stimulation was somewhat lower per pulse, and neither this uptake nor the preferential release of acetylcholine occurring at this frequency was significantly reduced by nifedipine. These findings suggest that the release of acetylcholine and VIP involve different VSCCs. The pattern of in vitro protein thiophosphorylation in tissue extracts of differentially stimulated myenteric neurons involved polypeptides of 205, 173, 86, 73, 57, 54, 46, 32, 28, and 24 kDa and was also markedly stimulus and nifedipine dependent. This suggests that different phosphoproteins are involved during the frequency-dependent activation of the different Ca^{2+} channels and exocytotic mechanisms. **Key Words:** Myenteric neurons—Differential frequency-dependent activation—Ca^{2+} channels—Transmitter release—Exocytosis—Protein phosphorylation. **Agoston D. V. and Lisziewicz J.** Calcium uptake and protein phosphorylation in myenteric neurons, like the release of vasoactive intestinal polypeptide and acetylcholine, are frequency dependent. *J. Neurochem.* **52,** 1637–1640 (1989).

The myenteric plexus–longitudinal muscle preparation (MPLM) of the guinea pig ileum (Paton and Zar, 1968) is one of the richest mammalian sources of cholinergic neurons, and it also contains considerable amounts of pharmacologically active peptides, among them vasoactive intestinal polypeptide (VIP), substance P, and somatostatin (Costa et al., 1986), located in distinct storage granules, all of which can be separated by high resolution density-gradient centrifugation in a zonal rotor (Dowe et al., 1980; Agoston et al., 1985). We have made use of this preparaiton to compare the intracellular dynamics of acetylcholine (ACh) and the storage and release of VIP (Agoston et al., 1985*a*, 1988), because the latter transmitter is often detected in cholinergic nerve terminals and can be regarded as a cholinergic cotransmitter (reviewed by Agoston, 1988). In brief, VIP release is markedly dependent on the supply of newly synthesized storage granules by axonal transport, whereas ACh-rich synaptic vesicles can be recycled in the terminal, and ACh release is independent of axonal transport, within the time scale of these experiments.

In a study (Agoston et al., 1988) of the frequency dependence of the stimulus-evoked release of ACh and VIP from MPLM it was found that such release was markedly frequency dependent, ACh being preferentially released at low frequencies (LF, 2–5 Hz) and VIP at high frequencies (HF, 20–50 Hz). The release induced by K^+ depolarization resembled that obtained at 8–10 Hz, a frequency that does not favor release of either of the transmitters.

In considering the possible basis for this inversely related frequency dependence of release it was thought that the exocytosis of the ACh-rich synaptic vesicles and VIP-rich dense-cored vesicles present in these neurons (Agoston et al., 1985*b*) might occur at different sites, involve different Ca^{2+} channels, and utilize different exocytotic mechanisms. We have, accordingly, studied the uptake of $^{45}Ca^{2+}$ and the pattern of protein thiophosphorylation in MPLM strips as a function of frequency. The analysis was facilitated by the use of a dihydropyridine derivative nifedipine, a selective blocker of one (the L type) of the two main types (N and L) of voltage-sensitive Ca^{2+} channels (VSCCs) known (Miller, 1987). Al-

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This work is dedicated to Prof. F. Cramer on the occasion of his 65th birthday.

Abbreviations used: ACh, acetylcholine; ATPγS, adenosine 5′-(γ-thio)triphosphate; cAMP, cyclic AMP; DTT, dithiothreitol; HF, high frequency; LDC, large, dense-cored; LF, low frequency; MPLM, myenteric plexus–longitudinal muscle preparation; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SEL, small recycling electrolucent; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate; VIP, vasoactive intestinal polypeptide; VSCC, voltage-sensitive Ca^{2+} channel.

though many other Ca^{2+}-channel blockers are known and could have been tested in this preparation, we felt that our preliminary results were of sufficient interest to merit publication at this stage.

MATERIALS AND METHODS

Tissue preparation, transmitter release, and Ca^{2+} uptake

Strips derived from MPLM preparations and weighing 50–150 mg were prepared from identical regions of the ileum of guinea pigs and subjected to supramaximal electrical field stimulation in a superfusion chamber, using square wave pulses in trains containing equal numbers of pulses at LF (5 Hz) and HF (50 Hz), or to depolarization by 60 m*M* KCl, as previously described (Agoston et al., 1985*a*, 1988). In some experiments nifedipine (Sigma; Munich, F.R.G.) was added to the perfusion medium at a final concentration of 10 μM. ACh and VIP release were measured and expressed as previously described (Agoston et al., 1988). Ca^{2+} uptake was measured, as described by Agoston et al. (1983) and Agoston and Kuhnt (1986), by adding $^{45}Ca^{2+}$ (specific radioactivity, 3 Ci · $mmol^{-1}$) to the perfusion medium in tracer amounts.

In vitro thiophosphorylation

Phosphorylation studies were performed on extracts of resting (control) or stimulated tissue by measuring the transfer of radioactive thiophosphoryl groups from [^{35}S]adenosine 5'-(γ-thio)triphosphate ([^{35}S]ATPγS). Stimulated strips were comminuted in an Ultraturrax blender in ice-cold 50 m*M* *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate (TES) buffer, pH 7.4, containing 0.4 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 0.01 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 $\mu g \cdot ml^{-1}$ leupeptin. The resulting 10% tissue suspension then was centrifuged in an Eppendorf centrifuge at 4°C for 5 min; following protein determination, in vitro thiophosphorylation was performed in the supernatant. The 25-μl reaction mixture contained (final concentrations): 50 m*M* TES, pH 7.4; 2.5 m*M* $MnCl_2$; 0.4 m*M* EDTA; 10 μM [^{35}S]ATPγS (specific activity, 0.2 $\mu Ci \cdot \mu M^{-1}$); and 15–30 $\mu g \cdot \mu l^{-1}$ of protein. In some experiments parallel samples were incubated in the presence of 10 μM nifedipine, or of a stable thio analogue of cyclic AMP (cAMP-Sp), or of 1.2 m*M* calcium. After incubations at 37°C, usually for 10 min, the reaction was terminated by adding sodium dodecyl sulfate (SDS) sample buffer and boiling for 5 min. The SDS-solubilized proteins were electrophoretically separated on a 12.5% SDS-polyacrylamide gel and Coomassie Blue-stained; after being destained, the gels were dried and autoradiographed for 2–4 days at room temperature, using Amersham Betamax x-ray film. The developed films were scanned in a Shimadzu densitometer, and the areas under the peaks were integrated. Basal incorporation into the various proteins was expressed as a percentage of the total radioactivity recovered, and incorporation after LF or HF stimulation was expressed as a percentage of the corresponding basal incorporations. Total trichloroacetic acid-precipitable ^{35}S incorporation was determined from small samples.

RESULTS

The activation of VSCCs, like transmitter release, is frequency dependent

Differential frequency dependence and pharmacology of $^{45}Ca^{2+}$ uptake. When MPLM strips were superfused with $^{45}Ca^{2+}$-containing Krebs–Ringer and LF or HF electrical field stimulation was applied, the kinetics of $^{45}Ca^{2+}$ uptake were found to be to some extent frequency dependent. Thus, even though after 5 min of stimulation the uptake of $^{45}Ca^{2+}$ had reached a plateau that was independent of stimulation frequency, HF $^{45}Ca^{2+}$ uptake had almost reached the plateau by 1 min but LF uptake had not quite reached it after 5 min (not shown). Given that the number of pulses delivered per train was identical, this indicated a difference in the kinetics of $^{45}Ca^{2+}$ uptake by differentially stimulated myenteric neurons. These differences became much more pronounced when the uptake of $^{45}Ca^{2+}$ took place in the presence of 10 μM nifedipine, a selective blocker of L-type Ca^{2+} channels. Nifedipine reduced the stimulated $^{45}Ca^{2+}$ uptake at 5 min during LF stimulation by only 10%, a barely significant value (Fig. 1; compare block 10 with block 9), but reduced it by as much as 60% during HF stimulation (Fig. 1; compare block 12 with block 11). The net uptake of $^{45}Ca^{2+}$ induced by K^+-stimulation also was reduced in the presence of nifedipine but only by ~30%. The unstimulated control uptake was not affected by nifedipine (not shown). These results suggest that at least two different VSCCs exist in MPLM preparations, one of which, probably an N-type channel, is preferentially activated at LF and the other, a nifedipine-sensitive L-type channel, is preferentially activated at HF.

Effect of L-channel blockade on transmitter release. Nifedipine resolved $^{45}Ca^{2+}$ uptake into two frequency-dependent processes, namely, uptake mediated by N-type channels preferentially activated by LF stimulation and L-type channels preferentially activated by HF stimulation. This finding strongly suggested that VIP release should be associated with

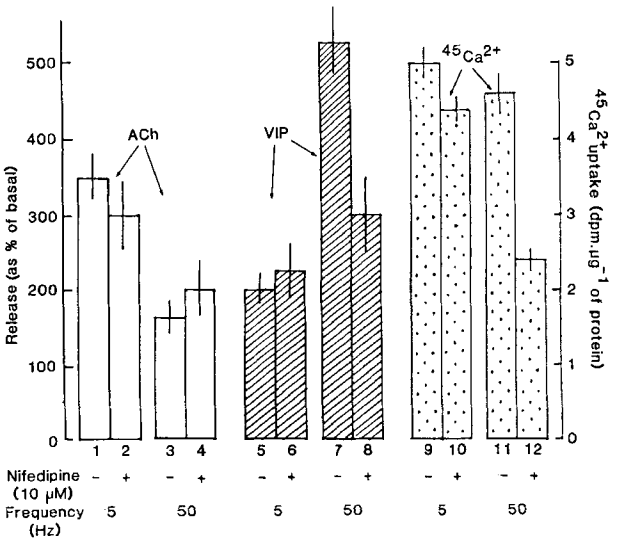

FIG. 1. The effect of the dihydropyridine derivative nifedipine, at a concentration of 10 μM, on the release of ACh (blocks 1–4) and VIP (blocks 5–8) and on the uptake of Ca^{2+} (blocks 9–12) by MPLM strips in response to LF (5 Hz) and HF (50 Hz) stimulation. The drug had little effect on any function at 5 Hz but at 50 Hz both VIP release and Ca^{2+} uptake were substantially depressed (compare block 8 with block 7, and 12 with 11). Note that ACh release is lower at HF than at LF (compare blocks 3 and 1), whereas that of VIP is enhanced (compare block 7 with block 5). The basal, non-stimulated release (g^{-1} of tissue · min^{-1}) of ACh was 106 ± 15 pmol (n = 18) and of VIP, 11 ± 2 fmol (n = 18). The basal uptake of $^{45}Ca^{2+}$ was 1 dpm · μg^{-1} of protein.

the activation of L-type channels and ACh release by the activation of N-type channels. This correlation was tested by studying the effect of nifedipine on ACh and VIP release. The results are also summarized in Fig. 1. Nifedipine was without effect on either the release of ACh (compare blocks 1 and 2) or the much lower release of VIP (compare blocks 5 and 6) induced by LF stimulation, but at 50 Hz it reduced the greatly augmented VIP release (compare block 7 with block 5) to close to the level found at LF (compare block 8 with blocks 7 and 5). By contrast, it had little or no effect on the reduced ACh output at HF (compare block 3 with blocks 4 and 1). In the case of stimulation with 60 m*M* K^+ (not shown), again there was little effect on ACh release but some effect on VIP release, the extent of which was consistent with equating the effect of high K^+ to stimulation at 8–10 Hz.

Variations in protein thiophosphorylation are induced in tissue extracts by differential stimulation in vivo

Three major proteins of molecular masses 173, 86, and 54 kDa and several minor proteins of molecular masses 205, 73, 57, 46, 32, 28 and 24 kDa were thiophosphorylated in tissue extracts of nonstimulated MPLM strips (see Table 1, column 2). The total in vitro incorporation of [^{35}S]ATPγS in extracts of LF-stimulated MPLM strips was accompanied by an increase in several phosphorylated protein bands (Table 1, column 3), especially the 205-, 57-, 54-, 46-, and 32-kDa bands, all of which showed a percentage increase greater than that of the total incorporation. By contrast, HF stimulation induced a lower total increase in incorporation (Table 1, column 4); however, the 205-, 73-, 54-, 46-, 32-, and 24-kDa proteins all showed a greater increase than that of the total incorporation. The 57- and 46-kDa bands showed much less thiophosphate incorporation and the 73- and 24-kDa bands much more compared with that after LF stimulation (Table 1, column 5). Addition of the stable cAMP thio analogue or Ca^{2+} did not significantly change the phosphorylation patterns induced by different frequencies of stimulation. As expected from the transmitter-release and $^{45}Ca^{2+}$-uptake studies, elevated potassium induced a phosphorylation pattern that was intermediate between those after LF and HF stimulation (not shown).

Nifedipine acts selectively on the L-type of VSCC, so the effect of 10 μ*M* nifedipine, during 50-Hz stimulation, on the pattern of thiophosphorylation observed following such stimulation was investigated. The main selective change in the pattern was an inhibition of the thiophosphorylation of the 24-kDa protein, bringing the concentration of the thiophosphorylated product closer to that it had achieved after 5-Hz stimulation. Because poststimulus thiophosphorylation probably represents a recovery process, this finding suggests that dephosphorylation of this protein may be involved in N-type channel function.

TABLE 1. *Effect of prestimulation on protein thiophosphorylation in strips of MPLM*

Peak (kDa)	Basal (% of recovered ^{35}S)	Increase over basal (as % of basal)		Ratio
		5 Hz	50 Hz	5 Hz/50 Hz
205[a]	3	**813**[a]	**225**	3.6[a]
173	18	109	47	2.3
86	40	97	42	2.3
73[b]	2	140	**220**[b]	0.6[b]
57[a]	4	**285**[a]	69	4.1[a]
54	15	**320**	**136**	2.3
46[a,c]	6	**369**[a]	**94**[c]	3.9[a,c]
32	1	**300**	**300**	1.0
28	4	0	−36	—
24[b]	7	132	**247**[b]	0.5[b]
Total incorporation of ^{35}S		179	84	2.1

Stimulation was at 5 or 50 Hz for 10 min. Bold figures in columns 3 and 4 are percentage increases relatively greater than the total increase.

[a,b] Components whose thiophosphorylation is specifically increased by prestimulation at [a]5 or [b]50 Hz relative to total.

[c] Raised when prestimulation at 50 Hz occurred in the presence of 10 μ*M* nifedipine.

DISCUSSION

Electrical signals reaching the nerve terminal via the axon open VSCCs, the L type of which is sensitive to dihydropyridine blockade but the N type is not (Miller, 1987). The resultant calcium influx triggers a sequence of events including phosphorylation of channel, vesicular, cytoskeletal, and receptor proteins followed by exocytosis and the release of transmitter (Reichardt and Kelly, 1983; Dunkley and Robinson, 1986; Armstrong and Eckert, 1987; Walker and Agoston, 1987; Augustine et al., 1987). The "classical" transmitter ACh is synthesized locally and stored in small recycling electrolucent (SEL) synaptic vesicles (reviewed by Whittaker, 1986). By contrast, peptide transmitters such as VIP are packaged, following perikaryal synthesis, into large, dense-cored (LDC) vesicles; these are then axonally transported in a fully loaded condition to the site of release (Nordmann, 1983). Differences in intracellular dynamics are also reflected in the finding that the classical transmitter is preferentially liberated at LF and the peptide at HF of electrical stimulation (Lundberg, 1981; Agoston et al., 1988). In the present work, we asked whether the differential frequency-dependent release of ACh and VIP from myenteric neurons can be correlated with a frequency-dependent activation of different VSCCs and if so, whether this is accompanied by different frequency-dependent patterns of protein phosphorylation.

A clear correlation has emerged between the frequency dependence of the activation of the two main types (L and N) of VSCCs and that of the release of ACh and VIP. This finding permits the following conclusion: the exocytosis of the VIP-containing LDC-vesicles is triggered primarily by the influx of Ca through L-type Ca^{2+} channels opened by HF stimulation, whereas that of the cholinergic SEL vesicles depends on the opening of N-type Ca^{2+} channels activated by LF stimulation. In neurons utilizing either ACh or VIP alone as transmitters, the two mechanisms can be visualized to exist in one tissue. However, for neurons utilizing both as cotransmitters, it would be necessary—as part of the functional consequences of transmitter coexistence (Bartfai et al., 1986)—to assume some form of topological separation of the two. Morphological studies have indeed indicated topological separation; the N-type channels appear to be concentrated at the active zones of the presynaptic membranes whereas the L-type are distributed on portions of the plasma membrane outside them (Miller, 1987). Also, exocytosis of LDC vesicles has been observed to occur peripherally to the active zones involved in the exocytosis of SEL vesicles (Zhu

et al., 1986). Other examples of a link between types of VSCC and the exocytosis of different types of storage particles are known; for example, the release of noradrenaline from sympathetic neurons and ACh from hippocampal slices both are dihydropyridine insensitive, whereas the release of substance P from dorsal root ganglia is dihydropyridine sensitive (Miller, 1987; Pohorecki et al., 1987; Hirning et al., 1988).

The use of [^{35}S]ATPγS for studying in vitro phosphorylation provides two advantages over the more frequently used [γ-^{32}P]ATP: (1) the thio analogue is an effective specific substrate of kinases but not of ATPases (Yasuoka et al., 1982; Cassel et al., 1983); and (2) the rate of dephosphorylation of proteins that have been thiophosphorylated is much slower than that of the ^{32}P-phosphorylated proteins (Cassel and Glaser, 1982). Taking advantage of thiophosphorylation results in the clear demonstration of frequency dependence in the pattern of stimulus-induced protein thiophosphorylation in the present work. Although the experimental design did not permit us to identify the phosphorylation of proteins according to their location (pre- or postsynaptic), it is tempting to relate these findings, together with the involvement of different VSCCs, to the different pathways of exocytosis activated by the application of two different frequencies.

Clearly, more work will be required to identify the role of the various protein kinase substrates and the significance of their poststimulus phosphorylation in transmitter release, but the fact that there are demonstrable frequency-dependent differences in the pattern of phosphorylation that can be modified by a Ca^{2+}-channel blocker provides a useful starting point for such investigations.

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