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Inhibition of Endogenous Acetylcholine Release by Blockade of Voltage-dependent Calcium Channels in Enteric Neurons of the Guinea-pig Colon

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Abstract—The effects on acetylcholine release from the guinea-pig colon of the N-type calcium channel blocker ω-conotoxin GVIA (ω-conotoxin), the L-type calcium channel blocker nifedipine and the putative blocker of T-type channels, flunarizine, have been investigated. Endogenous basal acetylcholine release and electrically (1 Hz, 1 ms, 450 mA)-evoked overflow in the presence of cholinesterase inhibitor were studied. ω-Conotoxin (1–10 nM) and nifedipine (0·03–3 μM) dose-dependently inhibited basal and electrically-evoked acetylcholine release. Maximal inhibition of basal or electrically-evoked acetylcholine release was about 40% for nifedipine and about 75% for ω-conotoxin. The potency of nifedipine was inversely related to the external calcium concentration: its EC50 value in low-calcium medium (0·5 mM) was as low as 12 nM. Flunarizine inhibited acetylcholine release only at concentrations higher than 0·2 μM. Our results are consistent with an involvement of N- and L-type calcium channels in the control of the endogenous acetylcholine release from the guinea-pig colon.

The role of different calcium channels in the control of cholinergic neurotransmission in the enteric nervous system is controversial. In the ileum of several species, the effect of the N-type calcium channel blocker ω-conotoxin GVIA (ω-conotoxin) is consistent with a major role of N-type channels in regulating acetylcholine release (Lundy & Frew 1988; Keith et al 1989; Yau et al 1989; De Luca et al 1990; Wessler et al 1990b). Conversely, the dihydropyridines, which are L-type calcium channel blockers, were ineffective in most studies (Kaplita & Triggle 1983; Lundy & Frew 1988; Agoston & Lisziewicz 1989; Wessler et al 1990b). However, low concentrations of dihydropyridines partially inhibited the electrically-evoked [^{14}C]acetylcholine (Bartfai & Vizi 1986) or endogenous acetylcholine release (Katsuragi et al 1990) from the guinea-pig ileum.

Compared with the ileum, the guinea-pig colon has received relatively little attention. In this organ, we observed inhibition of the endogenous acetylcholine release by L-type Ca^{2+}-channel blockers (Lecchini et al 1991). The aim of the present paper was to extend this observation by assessing the consequences of blockade of different voltage-dependent calcium channels on acetylcholine released from enteric neurons in the guinea-pig colon. To this end, we compared the effects of ω-conotoxin, nifedipine and the putative T-type channel blocker, flunarizine (Kaneda & Akaike 1989; Takahashi & Akaike 1991) on basal and electrically-evoked endogenous acetylcholine release. The calcium-dependence of the effect of nifedipine was also assessed.

Materials and Methods

Acetylcholine release

Endogenous acetycholine release from isolated preparations of distal colon taken from male guinea-pigs, 300–400 g, in the presence of the cholinesterase inhibitor physostigmine was studied according to Frigo et al (1984). Preparations (200–300 mg) were suspended isotonically under a tension of 10 mN in 3 mL organ baths containing modified Tyrode solution of the following composition (mM): 136·9 NaCl, 2·7 KCl, 1·04 $MgCl_2$, 1·8 $CaCl_2$, 11·9 $NaHCO_3$, 0·4 NaH_2PO_4, 5·5 glucose, 0·015 physostigmine sulphate, pH 7·3–7·4, gassed with 95% O_2–5% CO_2 and kept at 36·5°C. After an equilibration period of 60 min (the medium was changed every 20 min), the medium was collected (and immediately replaced) every 20 min until the end of the experiment and assayed for acetylcholine.

In stimulation experiments, six periods of electrical stimulation (biphasic square pulses; 1 Hz; 1 ms; 450 mA; 10 min) were applied every 20 min from t = 100 to t = 200 min. The 1 Hz frequency of stimulation was chosen because of its relative selectivity in stimulating the enteric cholinergic neurons (Cowie et al 1978).

Test compounds were added after the first two basal fractions or the first stimulation fraction had been collected. The effects of the test compounds were evaluated after a preincubation period of at least 20 min for each concentration. The calcium-dependence of basal acetylcholine release was checked after a 20-min preincubation period in Ca^{2+}-free solution. The dependence on external calcium of the effect of nifedipine was studied in modified Tyrode solution (0·5–4 mM $CaCl_2$). The variations in Ca^{2+} concentrations were made by varying $CaCl_2$ with no osmolar compensation. The effect of Ca^{2+}-free solution was evaluated after the 20-min preincubation.

At the end of each experiment, the preparation was blotted with filter paper and weighed.

Acetylcholine assay

Acetylcholine was assayed on the guinea-pig isolated ileum

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incubated in modified Tyrode solution with physostigmine sulphate (7·7 nM) and morphine sulphate (6·6 μM) added according to Paton & Vizi (1969). To allow for any effects that modifying drugs might have on the acetylcholine assay, the concentration of such drugs in the test samples were duplicated in the standard acetylcholine solutions during the assay. Each assay was performed in quadruplicate.

Drugs

Acetylcholine chloride; flunarizine dihydrochloride; physostigmine sulphate; ω-conotoxin GVIA (ω-conotoxin) were purchased from Sigma, St Louis, MO, USA, and morphine hydrochloride from SIFAC, Milano, Italy. Nifedipine was kindly supplied by Dr Thomas (Bayer, Milano, Italy). Nifedipine was dissolved in Tween 80 before dilution in distilled water. All solutions were protected from light and experiments were performed under yellow light.

Calculations and statistics

Endogenous acetylcholine release was expressed as ng (g tissue)$^{-1}$ min^{-1}. The basal release was calculated as the mean of the two basal fractions before stimulation. Electrically-evoked overflow was measured by subtraction of the calculated basal release from the total acetylcholine released in the stimulation fractions. In preliminary experiments, it was ascertained that acetylcholine release in 8 consecutive basal fractions or in 6 consecutive stimulation fractions did not significantly differ. Acetylcholine release in the 1st and 8th basal fraction was not significantly different when 6 electrical stimulation periods were applied in between. It was assumed that basal acetylcholine release remained constant during electrical stimulation.

The effects of test compounds on acetylcholine release were expressed as percentage variations with respect to the control values. For each preparation, the average acetylcholine release in the two first basal fractions or the calculated acetylcholine overflow in the first stimulation fraction was taken as control. Linear regression analysis was performed to calculate the drug concentrations producing half maximum effect (EC50) and the correlation coefficient (r) according to Tallarida & Murray (1987).

Statistical significance of differences between groups was analysed by Student's *t*-test for unpaired data.

Results

In 1·8 mM external Ca^{2+}, the average basal acetylcholine release was 18±3 ng (g tissue)$^{-1}$ min^{-1} (mean±s.e. of the first basal fractions, n=22). The electrically-evoked overflow was 48±8 ng (g tissue)$^{-1}$ min^{-1} (mean±s.e. of the overflow evoked by the first stimulation periods, n=20).

In Ca^{2+}-free medium, the basal acetylcholine release was inhibited by 79±7% (mean±s.e., n=3) and the electrically-evoked overflow by 92±5% (mean±s.e., n=3).

ω-Conotoxin-induced inhibition of acetylcholine release

ω-Conotoxin dose-dependently inhibited acetylcholine release, with EC50 values (with 95% confidence limits) of 2·10 (1·50–2·99) and 1·90 (1·05–2·64) nM against basal acetylcholine release and electrically-evoked overflow, respectively (Fig. 1).

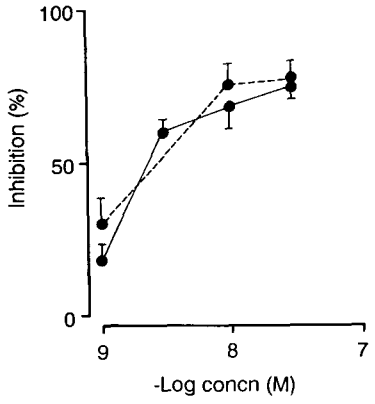

FIG. 1. Inhibition by ω-conotoxin of acetylcholine release from guinea-pig isolated colon. Log concentrations of ω-conotoxin are plotted against percentage inhibition of the acetylcholine basal release (——) or of the electrically (1 Hz, 1 ms, 450 mA, 10 min)-evoked overflow (- - -). Each point represents the mean (±s.e., n=6).

Nifedipine-induced inhibition of acetylcholine release

In 1·8 mM external calcium, nifedipine dose-dependently inhibited the basal acetylcholine release and the electrically-evoked overflow with EC50 values (with 95% confidence limits) of 0·34 (0·25–0·45) and 0·12 (0·09–0·16) μM, respectively.

The effect of nifedipine on basal acetylcholine release was calcium-dependent, as indicated by the correlation between the nifedipine EC50 values and the external Ca^{2+} concentrations in the 0·5–4 mM range ($r=0·997$, $P<0·05$, Fig. 2). In low (0·5 mM) and high (4 mM)-calcium medium, the calculated EC50 values were 12 (4–28) nM and 1 (0·42–2·16) μM.

Effect of combined ω-conotoxin and nifedipine

In separate experiments, we evaluated the effect of 1 μM nifedipine (proved to inhibit the basal/acetylcholine release by 37±5%, which did not differ from the 10 μM nifedipine-induced inhibition in control experiments: 43±4%,

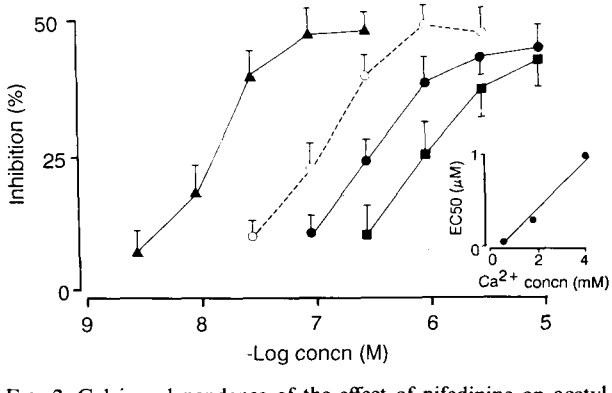

FIG. 2. Calcium-dependence of the effect of nifedipine on acetylcholine release from guinea-pig isolated colon. Log concentrations of nifedipine are plotted against percentage inhibition of the acetylcholine basal release (——) in 0·5 (▲), 1·8 (●) and 4 (■) mM external calcium or of the electrically (1 Hz, 1 ms, 450 mA, 10 min)-evoked overflow (○) in 1·8 mM external calcium. Each point represents the mean (±s.e., n=6). A significant correlation can be observed between external calcium and EC50 values (inset).

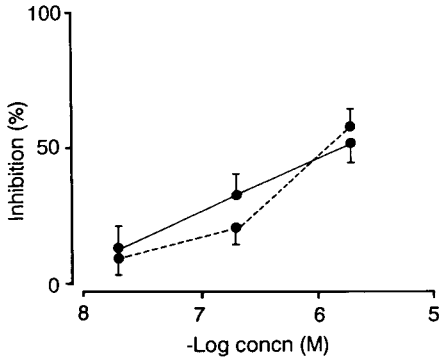

FIG. 3. Effect of flunarizine on acetylcholine release from guinea-pig isolated colon. Log concentrations of flunarizine are plotted against percentage inhibition of the acetylcholine basal release (——) or of the electrically (1 Hz 1 ms, 450 mA, 10 min)-evoked overflow (- - -). Each point represents the mean (±s.e., n=3).

mean±s.e., n=6) added to specimens incubated with a maximally effective ω-conotoxin concentration. In this set of experiments, inhibition of basal acetylcholine release by 30 nM ω-conotoxin was 75±7% (mean±s.e., n=4). When added to 30 nM ω-conotoxin, 1 μM nifedipine inhibited the ω-conotoxin-resistant acetylcholine release by 28±5% (mean±s.e., n=4). The overall inhibition of the basal acetylcholine release by combined 1 μM nifedipine and 30 nM ω-conotoxin was 81±4%.

Flunarizine

Flunarizine inhibited basal acetylcholine release and the electrically-evoked overflow only at concentrations higher than 0·2 μM (Fig. 3).

Discussion

The present findings show that up to 75% of the basal and electrically-evoked endogenous acetylcholine release from guinea-pig colon was inhibited by ω-conotoxin, while only a fraction (up to 45%) was sensitive to nifedipine. The effect of nifedipine was maintained in the presence of ω-conotoxin and was inversely related to the external calcium concentration.

The potency of ω-conotoxin in inhibiting the endogenous acetylcholine release in the guinea-pig colon agrees well with the potencies observed for inhibition of the cholinergic response to electrical stimulation in the same preparation (De Luca et al 1990) or in the guinea-pig bladder (Maggi et al 1988) and of [^{3}H]acetylcholine release from rat ileum and cortex (Wessler et al 1990b). Lower potency of ω-conotoxin was reported against the K^+-evoked endogenous acetylcholine release or the cholinergic response to electrical stimulation in the guinea-pig ileum (Lundy & Frew 1988; Keith et al 1989). Neurotransmitter release from different neurons or evoked by different stimuli can display variations in sensitivity to ω-conotoxin, possibly because of heterogeneity in N-type channels (Maggi et al 1988; De Luca et al 1990; Sher & Clementi 1991).

The potency of nifedipine in inhibiting endogenous acetylcholine release in our preparation is similar to its potency in the ileum (Katsuragi et al 1990). The calcium-dependence of the effect of nifedipine and the very high potency exhibited by this agent in the low-calcium medium (where the effect of changes in calcium permeability is maximized and any antagonism between external calcium and nifedipine is minimized) is consistent with an effect involving L-type calcium channels.

The effective concentrations of flunarizine in our preparation are unlikely to be specific for T-type channel blockade in neurons (Takahashi & Akaike 1991) and the observed effect could result from an interaction with N- or L-type channels or from calcium channel-unrelated effects (Gould et al 1983; Terada et al 1987).

Taken together, our results strongly suggest that opening of N-type calcium channels is required for the endogenous acetylcholine release from the guinea-pig colon. A minor role emerged also for the L-type channels, which appeared to control a fraction of the endogenous acetylcholine release, while no indication was obtained for a role of T-type channels.

Evidence for the presence of N- and L-type calcium channels in guinea-pig myenteric neurons rests on both binding and electrophysiological studies. Discrete high-affinity binding sites for ω-conotoxin and for dihydropyridines have been identified on the terminals of myenteric neurons (Ahmad et al 1989). In good agreement, two classes of calcium channels closely resembling the N- and L-type in other neurons (Hirning et al 1988) have been reported to account for the voltage-dependent calcium current in cultured myenteric neurons, while no T-type current appears to be present (Hirning et al 1990). At least the N-type current is likely to be related to the neurotransmitter release process in the cholinergic neurons of the small (Lundy & Frew 1988; Yau et al 1989; Wessler et al 1990b) or large intestine (De Luca et al 1990; present results). However, evidence is accumulating that L-type calcium channels may also control neurotransmitter release from myenteric intrinsic inhibitory, peptidergic (Bornstein et al 1985; Perney et al 1986; Agoston & Lisziewicz 1989) or cholinergic neurons (Katsuragi et al 1990; present results). While involvement of L-type channels in enteric peptidergic neurotransmission is not unexpected, the observed sensitivity of enteric acetylcholine release to L-type calcium channel blockade is difficult to explain in terms of a direct localization of L-type channels on cholinergic neurons on the basis of the current knowledge (Miller 1987).

Acetylcholine release from cholinergic neurons might result from activation of interneurons to a variable extent, depending on experimental conditions, animal species or tissue considered. Substance P, which releases acetylcholine from guinea-pig myenteric neurons (Yau et al 1989), is the best known transmitter whose release depends on L-type calcium channels (Miller 1987). Somatostatin-induced acetylcholine release is also inhibited by L-type calcium channel blockers in the guinea-pig intestine (Lu et al 1990). Thus, we can speculate that L-type currents might modulate neurotransmitter-induced acetylcholine release also in the myenteric plexus, as suggested in motor nerve endings or central neurons (Araujo et al 1990; Gray et al 1990; Wessler et al 1990a). Involvement of peptidergic interneurons could account for the effect of L-type calcium channel blockers in our experiments, although we cannot rule out a direct involvement of L-type channels in the acetylcholine release

process. In any case, our results are consistent with a major role being played by ω-conotoxin-sensitive, N-type calcium channels in the control of the endogenous acetylcholine release from myenteric neurons.

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