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CALCIUM CHANNEL BINDING IN NERVES AND MUSCLE OF CANINE SMALL INTESTINE

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SUMMARY Using [^{3}H]-nitrendipine (Nit) and [^{125}I]-omega conotoxin (w-CTX), the cellular and subcellular distribution of calcium channel subtypes in the homogenates of canine small intestinal circular muscle was studied. Nit. bound to the membranes from the circular smooth muscle cells (PM) and to the synaptosomal membranes from the deep muscular plexus (DMP); the Kd and Bmax values of Nit binding from these two sources were similar (Kd 0.4 ± 0.16 nM and 0.77 ± 0.24 nM; Bmax 206 ± 22 and 192 ± 39 fmol/mg of protein in DMP and PM respectively). w-CTX, however, bound only to the DMP (Kd 18.41 ± 7.5 pM, Bmax 265 ± 36 fmol/mg of protein). In DMP, nifedipine (10^{-6}M) failed to interact with the binding of w-CTX; similarly, no modulation of Nit binding with unlabelled w-CTX (10^{-7} M) could be detected. Therefore w-CTX and Nit binding sites represent two distinct, non-interactive and differentially distributed binding sites in canine small intestine.

INTRODUCTION Three main types of voltage-sensitive calcium channels (VSCC) have been recognized. The high threshold, long-lasting VSCC, the L-type, the low threshold transient, T-type and N-type which have properties in between these two types i.e. high threshold but transient in nature (1).

Dihydropyridines, benzothiazepines and phenylalkylamines are the classical agents that act relatively selectively on L-type of VSCCs; T- and N- types of VSCCs are generally insensitive to these agents (1). A polypeptide toxin from a fish-hunting snail, conus geographicus, omega-conotoxin GVIA (w-CTX) blocks N-channels and also some L-channels (2,3). Accordingly, L-subtypes of VSCCs have been further divided into Ln and Lm subtypes; Ln subtype being sensitive to w-CTX (4).

With a few exceptions (5,6) transmitter release from the presynaptic nerve terminals has been observed to be insensitive to the action of calcium channel blockers that act selectively on

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L-channels (for reviews see 1,4). w-CTX on the other hand, is a very potent blocker of the transmitter release in a number of systems (7-9). It has been hypothesized that the VSCCs responsible for the calcium entry into the presynaptic terminals required for the transmitter release are exclusively N-type, consequently, dihydropyridines are ineffective in blocking such a release. Indeed, radioactively labelled w-CTX has been demonstrated to bind to the brain synaptosomes (10,11). However, no detailed comparative binding studies using radioactively labelled ligands for N-channels (w-CTX) and L-channels (eg. dihydropyridines) on well defined membranes have been performed to localize the binding sites for these agents.

Recently we have described procedures to process the homogenates of the circular muscle of canine small intestine to obtain several fractions (12,13). One fraction was highly enriched by smooth muscle plasma membranes (13). Another fraction was found to contain a major proportion of intact synaptosomes from the deep muscular plexus (12). Other fractions of intermediate purity as well as some containing perhaps a majority of extrasynaptosomal neuronal membranes were also obtained. The purpose of the present study was to localize the binding sites for w-CTX and ^{3}H-nitrendipine in relation to the cellular and subcellular locus and to study the probability of interaction between these two types of binding sites.

MATERIAL AND METHODS Materials; [^{125}I]-omega-conotoxin (2200 ci/mmol) and [^{3}H]-nitrendipine (78.3 ci/mmol) were purchased from New England Nuclear Research Products, Boston (Ma). Unlabelled omega conotoxin GVIA was from Peninsula Laboratories, unlabelled nifedipine was from Sigma Chemical Company. All other salts and buffers were from Sigma.

Membrane preparation; Tissue handling and the membrane preparation from the circular smooth muscle of canine small intestine has been described in detail elsewhere (12,13). Briefly, the tissue obtained after careful dissection of the canine small intestine (13) was suspended in ten volumes of sucrose MOPS buffer [25 mM MOPS (3-[N-Morpholino]propanesulfonic acid), 10 mM $MgCl_2$, 8% sucrose; pH 7.4), was finely minced with scissors and was homogenized using a Polytron PT-20 homogenizer at a speed setting of 15,000 rpm for 21 sec (3 bursts of 7 sec each, allowing some time for cooling between the bursts). The scheme for the preparation has been described in detail elsewhere (12,13) and is presented in the fig.1.

Binding studies; The binding of ^{3}H-nitrendipine and ^{125}I-omega conotoxin (w-CTX) was performed in 50 mM Tris-HCl, pH 7.4 containing 0.2% bovine serum albumin. For the studies on the distribution of the binding sites, approximately 0.6 nM nitrendipine and 0.03 nM w-CTX was used. For saturation studies, the range of concentration used was 0.02 - 3 nM for nitrendipine

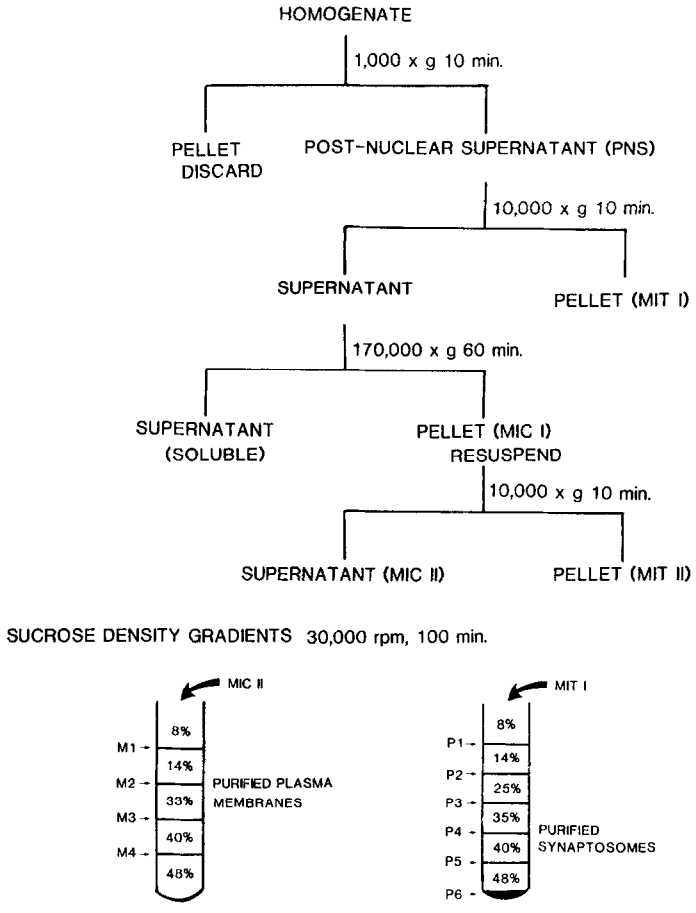

Fig. 1. Scheme of the preparation of membranes from the circular muscle of canine small intestine. The homogenate was centrifuged as indicated. Mit I and Mic II fractions were layered over the discontinuous sucrose density gradients and centrifuged at 30,000 rpm for 100 min. The purified plasma membranes (from circular smooth muscle) were obtained at 14-33% sucrose interface. The purified synaptosomes (from deep muscular plexus) were obtained at the interface of 35% and 40% sucrose.

and 0.02 - 1 nM for w-CTX. The non-specific binding was defined as that not displaced by 10^{-6} M unlabelled nifedipine and 10^{-6} M unlabelled w-CTX for nitrendipine and w-CTX binding studies respectively. Whatman GF/F glass fibre filters presoaked in the incubation buffer were used for nitrendipine binding and GF/B filters presoaked in 0.2% polyethylene imine in the incubation buffer were used for the w-CTX binding. No specific background in filters were present for nitrendipine binding. For w-CTX binding, however, depending on the concentration of the label, counts displaceable by unlabelled ligand were observed on the filters. Therefore, in every experiment, filter blanks were also included and the apparent specific binding observed to the filters was subtracted from each data point to obtain the actual value of the specific binding to the membranes.

The reaction was started by the addition of the membranes diluted in the incubation buffer (800 µL for nitrendipine and 200 µL for w-CTX). The total incubation volume for the nitrendipine

binding was 1 mL and for the w-CTX binding was 0.25 mL. Incubation was carried out at 25° C for 30 min. for nitrendipine and 45 min. for w-CTX, after which the samples were diluted with 4 mL ice cold incubation buffer, filtered under vacuum using the Millipore filtration apparatus followed by 2 x 4 mL washes for nitrendipine and 4 x 4 mL washes for the w-CTX binding.

The protein in the samples was measured by the method of Lowry (14).

RESULTS AND DISCUSSION Fig. 2 illustrates the distribution of nitrendipine and w-CTX binding to the fractions obtained after sucrose density gradient centrifugation of the microsomal fraction Mic II and the mitochondrial fraction Mit I. For the comparison, the values for the smooth muscle plasma membrane

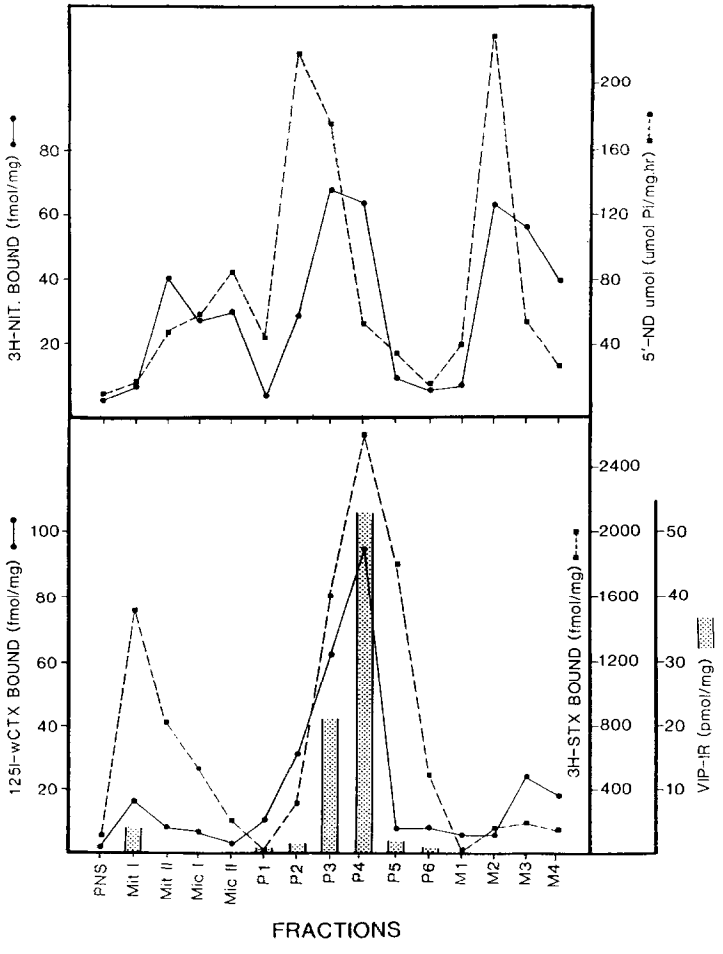

Fig. 2. The distribution of the markers, 5'-nucleotidase (5'ND), [^{3}H]-saxitoxin binding (3H-STX), VIP-immunoreactivity (VIP-IR) and the [^{3}H]-nitrendipine (3H-NIT) and [^{125}I]-omega conotoxin binding (125I-wCTX) to the fractions obtained during the differential and the sucrose density gradient centrifugation of the circular muscle of canine small intestine.

marker enzyme 5'-nucleotidase, neuronal marker ^{3}H-saxitoxin binding and the content of the vasoactive intestinal polypeptide-immunoreactivity (VIP-IR) as a marker of intact synaptosomes, as demonstrated in the previous studies (12) has also been included.

As is apparent from the distribution profile (Fig. 1b), only one peak for w-CTX binding was observed. This peak corresponds to the peak of VIP-IR content and the peak of ^{3}H-saxitoxin binding. Apparently w-CTX was binding mainly to the synaptosomal membranes. Relatively high w-CTX binding but low VIP-IR was also present in the fraction P2 which had high 5'-nucleotidase activity. Since there was no binding of w-CTX in the fraction M2, which was the fraction highly enriched in smooth muscle plasma membrane, the binding in P2 can not be attributed to the toxin binding to the smooth muscle plasma membranes. We believe that the binding observed in the fraction P2 is to the membranes from broken synaptosomes or the glial cells, which have a buoyant density similar to that of the plasma membranes from smooth muscle. This suggestion is supported by the relatively higher saxitoxin binding to this fraction as compared to the purified smooth muscle plasma membrane fraction M2.

Two maxima of nitrendipine binding were observed among the fractions. Both the fractions enriched in smooth muscle plasma membranes and in neuronal membranes contained a high density of nitrendipine binding sites. We studied the characteristics of nitrendipine binding to the plasma membranes and the neuronal membranes. As demonstrated by the saturation analysis of the binding data (table 1), these two sites were very similar in affinity for nitrendipine. Both of these sites represented a single population of non-interactive high affinity binding sites (Kd = 0.77 ± 0.24 (n=3) nM for the plasma membranes and 0.40 ± 0.16 nM (n=3) for the neuronal membranes), a conclusion based upon the linearity of the saturation plot when Scatchard transformation of the data was performed and on the value of the Hill slope (nH) which was near unity (nH = 0.98 ± 0.02 for the plasma membranes and 0.94 ± 0.05 for the neuronal membranes). The density of binding sites (Bmax) were also approximately the same in the two preparation (Bmax = 206 ± 22 in DMP and 192 ± 39 in PM).

The concentration-dependence of w-CTX binding on the deep muscular plexus synaptosomes also revealed a single population of binding sites with Kd and Bmax values of 18.41 ± 7.5 pM and 265 ±

TABLE I

SATURATION DATA FOR [^{3}H]-NITRENDIPINE AND [^{125}I]-OMEGA CONOTOXIN BINDING TO THE DEEP MUSCULAR PLEXUS AND THE CIRCULAR SMOOTH MUSCLE PLASMA MEMBRANES

	DMP		PM	
	NIT	CTX	NIT	CTX
Kd (nM)	0.40 ± 0.16	0.02 ± 0.01	0.77 ± 0.24	ND
Bmax (fmol/mg)	206 ± 22	265 ± 36	192 ± 39	
nH	0.94 ± 0.05	0.88 ± 0.03	0.98 ± 0.02	

The saturation parameters for the [^{3}H]-Nitrendipine (NIT) and [^{125}I]-omega conotoxin (CTX) binding to the deep muscular plexus (DMP) and smooth muscle plasma membranes (PM). The values given are the mean ± sd from three separate experiments performed in triplicate.

36 fmol/mg of protein respectively. The saturation data for both w-CTX and nitrendipine binding are summarized in table I.

Having observed that in deep muscular plexus synaptosomes, the binding sites for both w-CTX and nitrendipine were present, we examined the possibility of overlapping or interaction of the binding domains of these two ligands. Unlabelled w-CTX (10^{-7}M) did not displace any nitrendipine binding and 10^{-6} M unlabelled nifedipine did not displace any w-CTX binding (data not shown). These data support the notion that nitrendipine binding sites are distinct and non-interacting sites with those of w-CTX.

To our knowledge, this is the first demonstration of w-CTX binding to the peripheral (in this case enteric) nervous system and the first comparison of the distribution of various calcium channels in the membranes of the enteric nervous system and the gut smooth muscle. Effects of calcium channel blockers on enteric nerve function have not been analyzed. Neurotransmitter release from the synaptosomes prepared from various brain regions appear to be insensitive to the action of dihydropyridines (1,4) whereas w-CTX has been observed to be a potent blocker of such a release in many systems (7-9). A number of previous studies have demonstrated the presence of dihydropyridine binding sites in the synaptosomal preparation from neuronal cell lines or the brain regions. In the present study also we find substantial number of dihydropyridine binding sites. The involvement of the type/s of calcium channels in neurotransmitter release from the present

system needs to be studied in detail. Furthermore, since the neurotransmitter release from the presynaptic terminals in most of the cases does not depend upon the opening of L-channels, the significance of their presence on these structures remains to elucidated.

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