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IRREVERSIBLE MODIFICATION OF THE VOLTAGE-SENSITIVE CALCIUM CHANNEL BY *N*-ETHOXYCARBONYL-2-ETHOXY-1,2-DIHYDROQUINOLINE (EEDQ)

MURALI GOPALAKRISHNAN and DAVID J. TRIGGLE*
Department of Biochemical Pharmacology, School of Pharmacy,
State University of New York at Buffalo, Buffalo, NY 14260, U.S.A.

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Abstract—*N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) inhibited, *in vitro*, the specific binding of three structurally distinct L-type Ca^{2+} channel ligands, (+)[^{3}H]PN 200110, [^{3}H]desmethoxyverapamil and [^{3}H]*cis*-diltiazem to guinea pig ileal longitudinal smooth muscle. Maximum tension responses to Ca^{2+} in a K^+-depolarized functional smooth muscle preparation were reduced in a concentration-dependent manner following pretreatment with EEDQ and washout. Microsomal membranes prepared from smooth muscle pretreated with EEDQ followed by extensive washout showed a significant reduction in the amount of (+)[^{3}H]PN 200110 bound without change of ligand affinity. Similar results were obtained in cardiac ventricle microsomes. Preincubation with verapamil (1×10^{-5} M) largely prevented this reduction in [^{3}H]PN 200 110 binding sites by EEDQ. $^{45}Ca^{2+}$ uptake in cortical synaptosomes during 1-sec depolarization following 68.5 mM K^+ was also inhibited by EEDQ. Specific binding of [^{125}I]ω-conotoxin GVIA to rat cerebral cortex membranes was inhibited by EEDQ, also in an apparently irreversible manner as seen by the marked reduction in binding site density with no significant change in the K_D value. These observations indicate that EEDQ blocks Ca^{2+} channel function and reduces irreversibly both 1,4-dihydropyridine and ω-conotoxin GVIA binding sites.

Ca^{2+} influx through voltage-dependent Ca^{2+} channels is of particular importance to various cellular functions including excitation–contraction and stimulus–secretion coupling [1–3]. The L-type Ca^{2+} channels are sensitive to phenylalkylamines, benzothiazapines and 1,4-dihydropyridine activators and antagonists, whereas the N-type Ca^{2+} channels are sensitive to ω-conotoxin. Considerable evidence now exists to demonstrate that Ca^{2+} channels, especially the L-type, are subject to both homologous and heterologous regulatory influences during disease states and following cell lesions or chronic drug or hormone administration [4, 5]. The mechanisms of "up"- or "down-regulation" of Ca^{2+} channels are, however, less well understood. Alterations in channel expression and function presumably involve changes in the turnover of channel proteins.

A useful approach for studying the metabolism of certain classes of receptors has been the analysis of their reappearance after irreversible inactivation. *N*- Ethoxycarbonyl-2- ethoxy- 1,2-dihydroquinoline (EEDQ) is a carboxyl group activating agent used in peptide synthesis [6] which has proved to be a useful probe for the irreversible inactivation of several neurotransmitter receptors including those for norepinephrine (α- and β-adrenoceptors), acetylcholine (muscarinic), dopamine and serotonin [7–16]. The irreversible process presumably involves carboxyl group activation and subsequent cross-linking with nucleophilic groups at or adjacent to the receptor [17]. In this study, we have investigated the interaction of EEDQ at the L-type 1,4-dihydropyridine site and the N-type ω-conotoxin GVIA site associated with the voltage-dependent Ca^{2+} channel in ileal, cardiac and neuronal preparations.

* Address correspondence and reprint requests to: D. J. Triggle, Ph.D., School of Pharmacy, 126 Cooke Hall, State University of New York at Buffalo, Buffalo, NY 14260.

MATERIALS AND METHODS

Contractile responses. Male albino guinea pigs (Buckberg Farms, Tomkins Cove, NY), weighing 300–500 g, were killed by decapitation, and the terminal ileum was removed and placed in physiological saline solution (PSS) of the following composition (mM): NaCl, 118.0; KCl, 4.7; $NaHCO_3$, 25.0; KH_2PO_4, 1.0; $MgCl_2$, 1.0; $CaCl_2$, 1.8; and glucose, 10.0; aerated with O_2–CO_2 (95:5). Longitudinal muscle strips (1–2 cm length), prepared as described by Rosenberger *et al.* [18], were suspended under a resting tension of 0.5 g and allowed to equilibrate for 60 min in aerated (95% O_2:5% CO_2) PSS at 37° with changes of PSS every 15 min. Changes in tension were recorded isometrically using force displacement transducers (Grass FT03) and a Grass Polygraph (model 7B). After the initial equilibration period, tissues were incubated in Ca^{2+}-free PSS, prepared without the addition of $CaCl_2$, with several changes for 20 min. Tissues were then exposed to Ca^{2+}-free 80 mM K^+ (isotonic substitution of NaCl by KCl) depolarizing PSS for 10 min at the end of which time no response was apparent. Cumulative responses to calcium were then determined. Plateau responses were attained before addition of the next higher concentration of calcium. Following re-equilibration in PSS, tissues were treated with various concentrations of EEDQ for 60 min. After repeated

washout of the antagonist over a period of 15 min, a second cumulative concentration–response curve to Ca^{2+} was constructed. Geometric mean IC_{50} values were calculated. Control tissues, not exposed to EEDQ, were used to determine any time-dependent loss of sensitivity. Only one set of responses following antagonist treatment was determined in one tissue.

Radioligand binding. Binding assays were performed in microsomal membranes prepared from guinea pig ileal smooth muscle and heart and rat cerebral cortex according to the protocol extensively described and validated by Bolger *et al.* [19] and Janis *et al.* [20]. After incubation, membranes were filtered under vacuum with a Brandel cell harvester (model 24R, Brandel Instruments, Gaithersburg, MD) over glass fiber filters. Specific binding was defined as the difference between total and nonspecific binding. Inhibition of [^{3}H]PN 200110, [^{3}H]desmethoxyverapamil and [^{3}H]*cis*-diltiazem binding to ileal smooth muscle membranes was determined with six to nine concentrations of EEDQ. Radioligand binding assays were performed as described below.

[^{3}H]*PN* 200100. Specific [^{3}H]PN 200110 binding was determined as established previously in our laboratory [21]. Briefly, membrane protein (50–100 μg) was incubated with various concentrations of [^{3}H]PN 200110 in 5 mL Tris–HCl buffer (50 mM; pH 7.2) with and without the addition of 10^{-7} M unlabeled PN 200110 to a duplicate set of tubes for 90 min at 25°. [^{3}H]PN 200110 at a concentration of 5.52×10^{-11} M was used for competition binding assay. To determine the irreversible nature of EEDQ, ileal longitudinal muscle strips, 10–15 cm in length, were equilibrated in 50 mL PSS at 37° aerated with O_2:CO_2 (95:5) for 20 min. After initial equilibration, the strips were incubated with various concentrations of EEDQ for 60 min. Strips were then washed several times by exchanging similar volumes of aerated PSS maintained at 37° over a period of 15 min and transferred to ice-cold buffer for membrane preparation.

In vitro protection experiments were carried out by incubating guinea pig cardiac ventricle membrane homogenates at 25° for 15 min in the presence or absence of verapamil and then at 37° for 20 min with or without EEDQ. Samples were then dialysed (Spectra/por membrane tubing, cut off 12,000–14,000 mol wt) for 18 hr at 4° to remove drugs after which the B_{max} and K_D values of [^{3}H]PN 200110 binding were determined. We have shown previously this procedure to be an effective way of removing residual drugs [4]. Ileal microsomal membranes were not used because they underwent significant proteolytic degradation during dialysis.

[^{3}H]*Desmethoxyverapamil* ([^{3}H]*D*888). [^{3}H]D888 (0.882 nM) was incubated with ileal membrane protein (80–100 μg) and various concentrations of EEDQ in 1 mL of Tris–HCl buffer (50 mM; pH 7.2) for 120 min at 25°. Nonspecific binding was defined by the presence of 10^{-5} M verapamil. The samples were filtered through Whatman glass fiber GF/C filters soaked in 0.1% polyethylenimine and washed three times with 5 mL of ice-cold Tris buffer [22].

[^{3}H](cis)*(+)Diltiazem.* [^{3}H](*cis*)(+)Diltiazem (12.3 nM) was incubated with ileal membrane protein in Tris buffer as above, and nonspecific binding was defined by the presence of 10^{-4} M diltiazem [23].

[^{125}I]*ω-Conotoxin GVIA.* Specific binding of [^{125}I]ω-conotoxin to rat cerebral cortex membranes (4–5 μg protein) was determined by incubation in triplicate with [^{125}I]ω-conotoxin GVIA (1.26×10^{-13}–6.15×10^{-12} M) in 50 mM Tris–HCl buffer containing 0.1% bovine serum albumin in a 1 mL volume for 120 min at 37°. Nonspecific binding was determined by the inclusion of 10 nM unlabeled ω-conotoxin GVIA. Samples were filtered through GF/C filters presoaked in 0.3% polyethyleneimine and washed three times with 3-mL portions of ice-cold buffer. [^{125}I]ω-Conotoxin GVIA at a concentration of 1.42×10^{-12} M was used in competition binding assays. Changes in the toxin binding sites were examined by saturation analysis following extensive washout after pretreatment with EEDQ. Microsomal membranes were incubated with EEDQ at 37° for 20 min, and aliquots were diluted 4-fold with ice-cold buffer and centrifuged at 45,000 *g* for 20 min. This was followed by resuspension and three subsequent similar washes in 50 vol. of buffer, and the final pellet was resuspended in buffer for use in radioligand binding assays.

$^{45}Ca^{2+}$ *uptake.* Synaptosomes were prepared by the method of Hajos [24] from the cerebral cortex. The fast phase of $^{45}Ca^{2+}$ uptake was determined as in our previous study [25]. Synaptosomes were incubated at 37° for 15 min with various concentrations of EEDQ; then 50-μL aliquots containing 200–600 μg protein were added to the incubation medium at 37° containing $^{45}Ca^{2+}$, 5 mM K^+ (resting buffer) or 68.5 mM K^+ (isotonic substitution of choline chloride by KCl) in a final volume of 0.5 mL and were incubated for 1 sec. $^{45}Ca^{2+}$ uptake was terminated by the addition of 4 mL of ice-cold Ca^{2+}-free resting buffer containing 3 mM ethyleneglycolbis(aminoethylether)tetraacetate (EGTA). This solution was then filtered over Whatman GF/B filters and washed four times with portions of ice-cold resting buffer. Net $^{45}Ca^{2+}$ uptake was determined [25] as the difference between uptake in the resting buffer (5 mM K^+) and that in depolarizing buffer (68.5 mM K^+). The effects of EEDQ to inhibit [^{3}H]PN 200110 binding to synaptosomes were also assayed in the same preparation, save that all binding incubations were carried out in a 5 mM K^+ buffer. [^{3}H]Radioligand binding to filters was determined by liquid scintillation spectrometry and [^{125}I]ligand binding by gamma counting. Protein was determined by the method of Lowry *et al.* [26] using bovine serum albumin as the standard.

B_{max} and K_D values of radioligand binding were determined by analysis of the saturation data [27] with an iterative nonlinear LIGAND-based curve-fitting program implemented on an IBM PS/2 computer. Analysis of pharmacologic data employed standard pharmacologic programs [28]. Significant differences between groups of means were assessed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple range test using the SPSS/PC+ system. Results were considered statistically significant at $P(F) < 0.05$ by ANOVA combined with $P < 0.05$ for the Newman–Keuls test. All values are expressed as means ± SE.

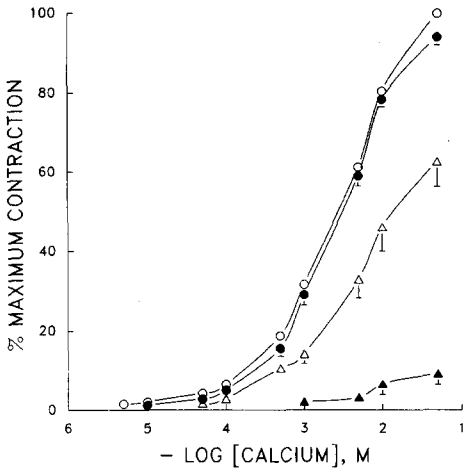

Fig. 1. Inhibition of responses of depolarized (80 mM K^+) guinea pig ileal smooth muscle to Ca^{2+} by EEDQ. Control (○) EC_{50}, 1.12×10^{-3} M (95% confidence limits (C.L.) 0.88–1.41 $\times 10^{-3}$ M); EEDQ: 0.5×10^{-4} M (●) EC_{50}, 1.43×10^{-3} M (95% C.L. 0.87–2.34 $\times 10^{-3}$ M); 1×10^{-4} M (△) EC_{50}, 2.40×10^{-3} M (95% C.L. 1.07–5.42 $\times 10^{-3}$ M); 2×10^{-4} M (▲) EC_{50}, 2.45×10^{-2} M (95% C.L. 1.04–5.78 $\times 10^{-2}$ M). Values are means ± SE of six observations.

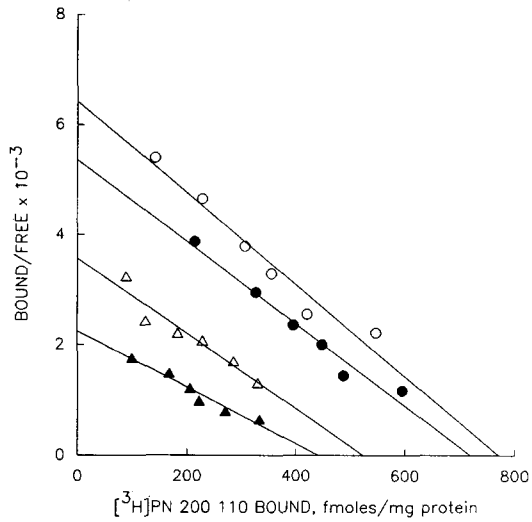

Fig. 2. Scatchard representation of inhibition of specific [^{3}H]PN 200 110 binding by EEDQ. Control binding (○) B_{max}, 779 ± 29 fmol/mg; K_D, 0.09 ± 0.02 $\times 10^{-9}$ M; binding in the presence of EEDQ: 1×10^{-4} M (●) B_{max}, 704 ± 109 fmol/mg; K_D, 0.08 ± 0.01 $\times 10^{-9}$ M; 5×10^{-4} M (△) B_{max}, 478 ± 46 fmol/mg; K_D, 0.11 ± 0.02 $\times 10^{-9}$ M; 8×10^{-4} M (▲) B_{max}, 349 ± 80 fmol/mg; K_D, 0.12 ± 0.04 $\times 10^{-9}$ M (means ± SE, N = 3–5). The data shown are from representative experiments.

(+)[^{3}H]PN 200 110 [isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridine carboxylate; sp. act. 72.6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels], [^{3}H]-(−)*cis*(+)-diltiazem (60.7 Ci/mmol), [^{125}I]ω-conotoxin GVIA (2200 Ci/mmol) and $^{45}CaCl_2$ (4–50 Ci/g) were purchased from DuPont–New England Nuclear (Boston, MA). [^{3}H]-(−)D888 (68 Ci/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL). EEDQ was purchased from the Aldrich Chemical Co. (Milwaukee, WI); other chemicals from Fisher Scientific or the Sigma Chemical Co. (St. Louis, MO) were of the highest purity routinely available. Stock solutions of EEDQ (10^{-1} M) were freshly prepared in ethanol immediately before use.

RESULTS

Contractile responses in ileal smooth muscle. The cumulative concentration–response curve to Ca^{2+} in Ca^{2+}-free K^+-depolarizing medium in guinea pig ileal smooth muscle was shifted to the right and the maximum response decreased by EEDQ in a concentration-dependent manner (Fig. 1). An approximately 50% reduction in the maximum tension response elicited by Ca^{2+} (2.8 ± 0.19 g in this preparation) could be obtained after pretreatment with 1.1×10^4 M EEDQ.

Radioligand binding. The ability of EEDQ to compete for both the L- and N-type calcium channel binding sites was investigated. When added directly to binding assays, EEDQ inhibited the specific binding of [^{3}H](−)D888, [^{3}H](*cis*)-(+)diltiazem and [^{3}H]PN 200 110 to the ileal smooth muscle membranes with IC_{50} values (means ± SE, N = 3–5) of $1.26 \pm 0.22 \times 10^{-4}$ M, $2.41 \pm 0.61 \times 10^{-4}$ M and $3.19 \pm 0.77 \times 10^{-4}$ M respectively. [^{125}I]ω-Conotoxin GVIA binding to rat cerebral cortex membranes was inhibited by EEDQ with a similar IC_{50} value ($1.96 \pm 0.73 \times 10^{-4}$ M).

Binding of (+)[^{3}H]PN 200 110 to the microsomal membrane fraction of guinea pig ileal muscle was saturable, $B_{max} = 779 \pm 29$ fmol/mg protein and $K_D = 0.09 \pm 0.02 \times 10^{-9}$ M. When ileal strips were incubated with EEDQ and then thoroughly washed, the preincubation with EEDQ prevented subsequent binding of [^{3}H]PN 200 110 to their sites. As shown in Fig. 2, the decrease in specific binding following pretreatment with EEDQ was characterized by a concentration-dependent reduction in B_{max}. No significant change in the K_D value of (+)[^{3}H]PN 200 110 binding was observed. An alternate protocol involving the incubation of ileal microsomal membranes with EEDQ (10^{-3} M) for 20 min at 37° followed by recentrifugation at 50,000 *g* for 15 min and resuspension, three times, gave a similar reduction in B_{max} of 63% from 709.7 ± 104.1 fmol/mg protein (N = 3) to 260.2 fmol/mg protein (N = 2) without significant change in K_D value (control, $9.6 \pm 2.8 \times 10^{-11}$ M; EEDQ-treated, $1.3 \pm 0.7 \times 10^{-10}$ M).

Similar observations were made in cardiac ventricle microsomes where incubation of membrane homogenates with 10^{-3} M EEDQ followed dialysis to remove the drug resulted in a significant reduction (55%) in [^{3}H]PN 200 110 binding site density from control, 236 ± 21 fmol/mg to 107 ± 26 fmol/mg protein without significant change in the K_D value (control, $6.9 \pm 1.9 \times 10^{-11}$ M and EEDQ-treated, $7.2 \pm 1.4 \times 10^{-11}$ M; N = 3). Partial protection against the actions of EEDQ was provided by prior

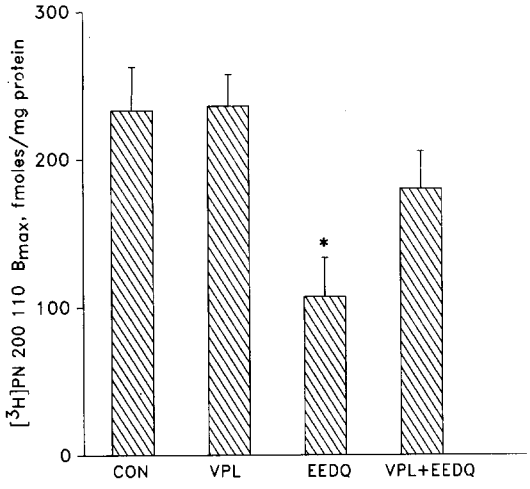

Fig. 3. Partial protection of [^{3}H]PN 200 110 binding sites from EEDQ following prior incubation with 1×10^{-5} M verapamil. B_{max} values for control membranes before dialysis (CON), membranes after dialysis treated with verapamil (VPL), 10^{-3} M EEDQ alone (EEDQ), or verapamil and EEDQ (VPL + EEDQ) are shown as means ± SE of three separate experiments. Key: (*) significantly ($P < 0.05$) less than control as determined by one-way analysis of variance and the Neumann–Keuls multiple range test.

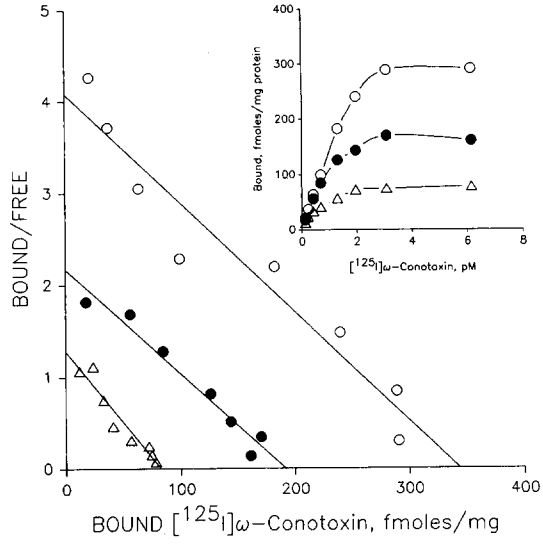

Fig. 4. Scatchard representation of the inhibition of specific [^{125}I]ω-conotoxin GVIA binding to rat cerebral cortex membranes by various concentrations of EEDQ. Inset: Saturation curves for specific [^{125}I]ω-conotoxin GVIA binding in the absence (○) and presence [(●) 5×10^{-4} M; (△) 10^{-3} M] of EEDQ. Membranes were incubated with EEDQ for 20 min at 37°, washed three times by centrifugation, and then incubated with concentrations of [^{125}I]ω-conotoxin GVIA ± 10^{-8} M unlabeled ω-conotoxin GVIA for 120 min at 37°. The data shown are representative of results obtained from four separate experiments for control [(○) B_{max}, 350 ± 31 fmol/mg; K_D, 0.39 ± 0.05 × 10^{-12} M] and following pretreatment with 5×10^{-4} M EEDQ [(●) B_{max}, 189 ± 18 fmol/mg; K_D, 0.30 ± 0.04 × 10^{-12} M] and 10^{-3} M EEDQ [(△) B_{max}, 89 ± 5 fmol/mg; K_D, 0.31 ± 0.01 × 10^{-12} M]. B_{max} values after EEDQ treatment were significantly ($P = 0.05$) different from control.

treatment with antagonist. Preincubation of cardiac microsomes with 1×10^{-5} M verapamil prior to EEDQ revealed a partial, but significant protection of [^{3}H]PN 200 110 binding site blockade elicited by EEDQ (B_{max}, 180 ± 25 fmol/mg; K_D, 1.4 ± 0.25 × 10^{-10} M; N = 3, Fig. 3).

[^{125}I]ω-Conotoxin binding. Saturation analysis of [^{125}I]ω-conotoxin binding to rat cerebral cortex membranes treated with EEDQ (5×10^{-4} M and 10^{-3} M) for 20 min at 37° followed by extensive washing resulted in a concentration-dependent reduction in the B_{max} of [^{125}I]ω-conotoxin binding sites (Fig. 4). No significant difference in the affinity of binding sites was observed between control and membranes treated with EEDQ.

Effect of $^{45}Ca^{2+}$ uptake. We also examined whether EEDQ antagonizes Ca^{2+} entry through voltage-dependent calcium channels in the nerve terminals. The fast component of $^{45}Ca^{2+}$ uptake (1 sec) into guinea pig cortical synaptosomes following K^+ depolarization was reduced in a concentration-dependent manner by EEDQ (Fig. 5). Significant reductions of $^{45}Ca^{2+}$ uptake occurred at concentrations of EEDQ ≥ 100 μM. The IC_{50} value for EEDQ antagonism of $^{45}Ca^{2+}$ uptake was estimated to be 9.8×10^{-5} M. This concentration of EEDQ that reduced K^+-stimulated $^{45}Ca^{2+}$ uptake by 50% was very similar to the concentration that inhibited half-maximal [^{3}H]PN 200 110 binding to the synaptosomal preparation (1.06 ± 0.15 × 10^{-4} M).

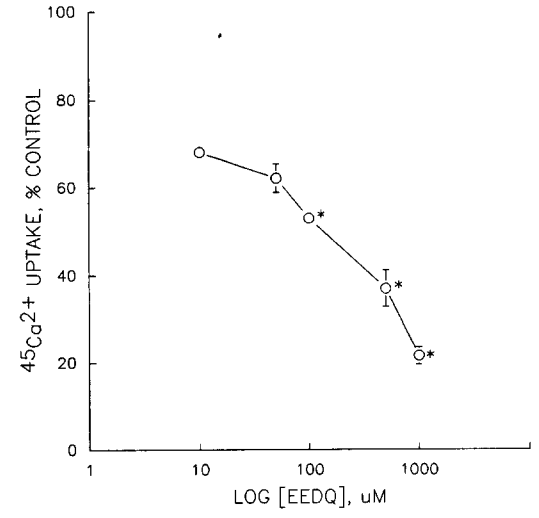

Fig. 5. Effect of EEDQ on $^{45}Ca^{2+}$ uptake by guinea pig cortical synaptosomes during 1-sec depolarization with 68.5 mM KCl. Values are means ± SE of at least four experiments, each done in quadruplicate. Key: (*) significantly less than control ($P < 0.05$). Control value of $^{45}Ca^{2+}$ uptake: 2 nmol/mg.

DISCUSSION

EEDQ has been shown to modify irreversibly

several receptors including α-adrenergic [7, 8], β-adrenergic [9], dopaminergic [10–13], muscarinic [14, 15] and serotoninergic [16] receptors and ion channels, including the sodium channel [29, 30]. This study presents evidence that EEDQ inhibits irreversibly 1,4-dihydropyridine binding sites associated with the voltage-dependent Ca^{2+} channels in guinea pig ileal smooth muscle *in vitro*, as well as ω-conotoxin GVIA binding sites in rat cerebral cortical membranes, and reduces depolarization-induced $^{45}Ca^{2+}$ uptake in guinea pig cortical synaptosomes.

K^+-Depolarization-induced tension responses in ileal smooth muscle are highly dependent on extracellular Ca^{2+} influx through the L-class of 1,4-dihydropyridine-sensitive voltage-dependent Ca^{2+} channels [31]. The marked reduction in responses to Ca^{2+} in K^+-depolarizing medium following pretreatment with EEDQ is consistent with an interaction of EEDQ at the voltage-dependent Ca^{2+} channel. This is supported by the inhibition of specific (+)[^{3}H]PN 200 110 binding after incubation *in vitro*, with EEDQ and the partial protection of this inhibition by verapamil. Although it has been shown that EEDQ interacts with other receptor systems [7–16], this study shows that voltage-dependent calcium channels represent other sites for irreversible interaction.

The fast component of $^{45}Ca^{2+}$ uptake, generally assumed to be mediated by a voltage-dependent Ca^{2+} channel insensitive to 1,4-dihydropyridine ligands [32], was also inhibited by EEDQ at concentrations that inhibited synaptosomal (+)[^{3}H]PN 200 110 binding. That EEDQ inhibited the fast phase of synaptosomal $^{45}Ca^{2+}$ uptake suggests that it may also interact with other voltage-dependent channel types including the ω-conotoxin-sensitive N-type Ca^{2+} channels. This was confirmed by our observations that EEDQ inhibited irreversibly ω-contoxin binding in cerebral cortex membranes.

The lack of ready reversibility of EEDQ inhibition of tension responses, and of (+)[^{3}H]PN 200 110 and of [^{125}I]ω-conotoxin binding, is consistent with covalent modification of the Ca^{2+} channel, presumably through the mixed anhydride pathway [17]. However, the nature of this site is not established by the present experiments. A potential site for irreversible interaction at the voltage-dependent Ca^{2+} channel will include the ligand binding α_1 subunit of the L-type channel which has been shown to be necessary for channel function [33]. The obvious possibility exists, however, that EEDQ interacts at sites other than the voltage-dependent Ca^{2+} channel and at which Ca^{2+} channel function is modified. Additionally, EEDQ may modify other ion channel types including K^+ channels as well as modify events subsequent to Ca^{2+} channel activation that are associated with response generation. Nonetheless, EEDQ may prove to be an irreversible probe suitable for studying voltage-dependent Ca^{2+} channel metabolism and function. Further studies are in progress.

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