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Characterization and modulation of [^{125}I]iberiotoxin-D19Y/Y36F binding in the guinea-pig urinary bladder

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Abstract

The radioligand binding characteristics of the Ca^{2+}-activated K^+ channel ligand [^{125}I]iberiotoxin-D19Y/Y36F were examined in guinea-pig urinary bladder membranes. Saturation analysis revealed a single class of high affinity binding sites in the bladder with a K_D value of 45.6 pM and a B_{max} value of 112 fmol/mg protein. Specific binding was displaced by unlabeled iberiotoxin and penitrem A, but not by blockers of other classes of K^+ channels including α-dendrotoxin, margatoxin and apamin. The indole alkaloids, paxilline and verruculogen, significantly increased binding by 4.5- and 4.3-fold, respectively. Tetraacetic acid derivatives such as ethylenediamine tetraacetic acid and ethyleneglycoltetraacetic acid enhanced specific [^{125}I]iberiotoxin-D19Y/Y36F binding about 2.5-fold, which was not attributable to calcium chelation. This increase was due to a significant change in ligand binding affinity (K_D = 6.3 pM), but not due to a change in the B_{max}, indicating that these compounds may enhance toxin binding via allosteric interactions. Collectively, these results demonstrate that the binding sites for [^{125}I]iberiotoxin-D19Y/Y36F present in the urinary bladder shows a pharmacological profile typical of maxi-K^+ channels and can be modulated, not only by previously known indole alkaloids, but also by tetraacetic acid analogs.

Keywords: Maxi-K^+ channel; Radioligand binding; Iberiotoxin; Urinary bladder

1. Introduction

Various types of K^+ channels — voltage gated, Ca^{2+}-activated and ATP-sensitive — have been shown to modulate spontaneous electrical and mechanical activity in the detrusor smooth muscle (Brading et al., 1996). In particular, the large conductance Ca^{2+}-activated K^+ (maxi-K^+) channels present in bladder detrusor cells (Klöckner and Isenberg, 1985) play a fundamental role in controlling cell excitability and contractility (Stuarez-Kurtz et al., 1991). Both membrane depolarization and intracellular Ca^{2+} elevations subsequent to either influx or intracellular release activate these channels (Imaizumi et al., 1996). Studies have shown that these channels play a fundamental role in maintaining the resting membrane potential, and significantly contribute to the ionic current underlying repolarization and after-hyperpolarization of action potentials in detrusor smooth muscle. Iberiotoxin, a very specific peptidyl blocker of high-conductance Ca^{2+}-activated K^+ channels, has been shown to increase the electrical activity of smooth muscle bladder cells by depolarizing the membrane potential several millivolts and by increasing the frequency of spontaneous action potentials (Heppner et al., 1997).

Modulation of maxi-K^+ channels may represent an opportunity for the development of agents to control smooth muscle dysfunction such as detrusor instability. Several putative activators of maxi-K^+ channels have been described including the benzimidazolones such as NS004, NS1619, and niflumic acid (Edwards et al., 1994; Gribkoff et al., 1996). However, these compounds are, in general, not very potent or selective to serve as valuable pharmacological probes. Recently, ZD6169, an anilide tertiary carbinol with K_{ATP} channel activator properties and with potential for the management of urinary urge incontinence

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(Howe et al., 1995; Trivedi et al., 1995), has been shown to activate maxi-K^+ channels in the bladder *albeit* at higher concentrations (50 μM; Hu and Kim, 1997). Radioligand binding studies with [^{125}I]charybdotoxin have identified several maxi-K^+ channel modulators including glycosylated triterpenes (dehydro-soyasaponin-I) and several indole diterpene alkaloids such as paxilline, verruculogen, penitrem A and aflatrem (reviewed in Kaczorowski et al., 1996). Although [^{125}I]charybdotoxin has served as a useful tool to study maxi-K^+ channels, this ligand, in addition, interacts with high affinity to several voltage-gated K^+ channels and small and intermediate conductance Ca^{2+}-activated K^+ channels present in neurons, smooth muscle and lymphocytes (Vàzquez et al., 1989, 1990; Deutsch et al., 1991). More recently a modification of the more selective ligand, iberiotoxin, has been synthesized ([^{125}I]iberiotoxin-D19Y/Y36F) and shown to bind with high affinity to maxi-K^+ channels in trachea smooth muscle (Koschak et al., 1997). In this study, we have characterized large conductance Ca^{2+}-activated K^+ channels in the guinea-pig urinary bladder by assessing the pharmacology and modulation of [^{125}I]iberiotoxin-D19Y/Y36F binding.

2. Materials and methods

2.1. Materials

Unlabeled iberiotoxin-D19Y/Y36F iodinated with carrier free Na^{125}I using the lactoperoxidase technique, was obtained from NEN Life Science Products (Boston, MA). The product was purified using a C-18 reverse phase column (Zorbax SB 300A C-18, 4.6 mm × 25 cm) with a linear gradient from 19% to 30% acetonitrile in 0.1% trifluoroacetic acid and the radioiodinated peptide diluted and stored in 100 mM Tris buffer (pH, 7.5) containing 0.1% bovine serum albumin at −20°C. Unlabeled iberiotoxin, NS1619 and 1,2-bis(2-amino phenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA) were purchased from Research Biochemicals (Natick, MA). α-Dendrotoxin, margatoxin, mast cell degranulating peptide and penitrem A were purchased from Alamone Labs (Jerusalem, Israel). Verruculogen, ethylenediamine tetraacetic acid disodium salt (EDTA), ethyleneglycoltetraacetic acid (EGTA) and niflumic acid were purchased from Sigma (St. Louis, MO). Apamin was purchased from Calbiochem (La Jolla, CA). (−)-Cromakalim and ZD6169 were synthesized at Abbott Laboratories (Abbott Park, IL). All other reagents were obtained from commercial sources.

2.2. Membrane preparation

Animals were maintained according to the protocol approved by Abbott's Institutional Animal Care and Use Committee. Briefly, bladders were dissected from male guinea-pigs (250–300 g; Hartley, Charles River, Wilmington, MA) and homogenized in ice-cold buffer (Tris 25 mM; phenylmethylsulfonylfluoride 0.1 mM; pH = 7.2) using a Polytron homogenizer. The homogenate was filtered through cheesecloth and then centrifuged at 40,000 × *g* for 15 min at 4°C. The pellet was resuspended in ice-cold buffer and used for radioligand binding. In some experiments, membranes were resuspended in EDTA (1 mM), incubated for 30 min on ice, centrifuged and washed twice with ice-cold buffer by repeated centrifugation prior to binding studies.

2.3. Radioligand binding

[^{125}I]iberiotoxin-D19Y/Y36F binding was carried out by incubation in a final volume of 500 μl using about 10–20 μg protein per tube at room temperature. For saturation experiments, membranes were incubated in assay buffer (Composition, mM: Tris 25; phenylmethylsulfonylfluoride 0.1 and bovine serum albumin 0.1%; pH = 7.2) with or without EDTA 1 mM and increasing concentrations of radioligand (0.5–150 pM). Incubations were carried out for either 2.5 or, in some experiments, for 7 h. Specific binding was defined with unlabeled iberiotoxin (100 nM). For association kinetics of [^{125}I]iberiotoxin-D19Y/Y36F binding, membranes were incubated with about 20 pM of radioligand for various time intervals. For dissociation studies, membranes were first incubated with 20 pM radioligand for 2.5 h followed by the addition of 100 nM iberiotoxin for various intervals of time to initiate dissociation. In competition experiments, membranes were preincubated with varying concentrations of compounds for 2 h followed by an additional incubation for 2.5 h in the presence of [^{125}I]iberiotoxin-D19Y/Y36F (8 pM). Incubations were terminated by rapid vacuum filtration over GF/B glass fiber filters presoaked in polyethyleneimine 0.5%, and filters washed three times with 1.5 ml of ice-cold 50 mM Tris buffer (pH = 7.2). Bound radioactivity was quantitated by gamma counting spectroscopy at an efficiency of 80%. Protein was determined using bovine serum albumin as the standard (Lowry et al., 1951). All dilutions of radioligand and compounds were prepared in the assay buffer containing Tris 25 mM, phenylmethylsulfonylfluoride 0.1 mM and bovine serum albumin 0.1% with or without EDTA 1 mM (pH = 7.2).

2.4. Data analysis

The binding parameters (K_D and B_{max}), kinetic data and potency values were obtained from nonlinear regression analysis using Graphpad Prism software (San Diego, CA). The K_I values for unlabeled drugs were calculated from the concentration dependence of inhibition using the equation, $K_I = IC_{50}/(1 + ([\text{radioligand}]/K_D)$ (Cheng and Prusoff, 1973). For kinetic analysis, data points were fitted

to monophasic exponential association and dissociation equations respectively. All values are expressed as means ± S.E. of *n* individual experiments conducted using separate batches of membrane preparations. Significant differences between groups of means were assessed by the unpaired Student's *t*-test.

3. Results

3.1. Characteristics of [^{125}I]iberiotoxin-D19Y / Y36F binding

Over the radioligand concentration range examined (0.5–150 pM), specific [^{125}I]iberiotoxin-D19Y/Y36F binding was detected in guinea-pig urinary bladder membranes. As shown in Fig. 1, binding was saturable and Scatchard analysis of the data yielded a B_{max} value of 112 ± 21 fmol/mg protein and a K_D value of 45.6 ± 6.9 pM ($n = 9$). Similar K_D values were obtained when incubations were carried out for an extended period (7 h) to further ensure that equilibrium conditions were achieved (data not shown). It should be noted that these studies were carried out in the absence of EDTA (see below).

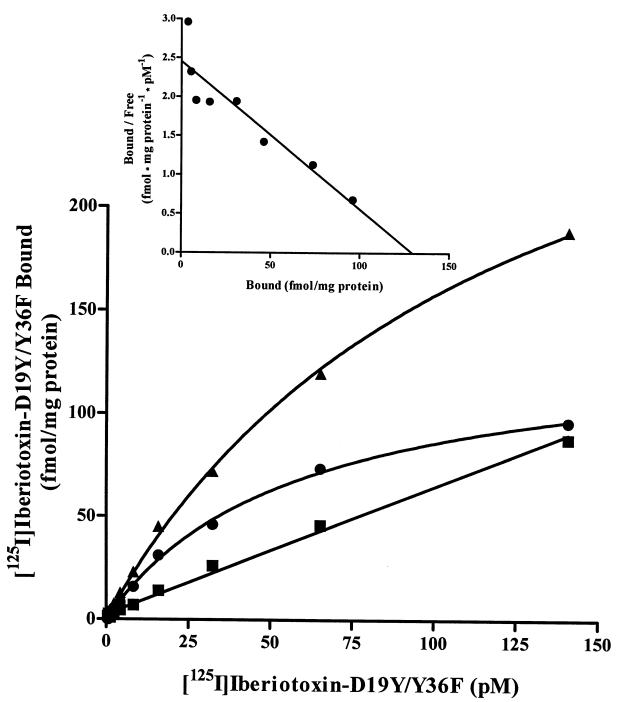

Fig. 1. Binding of [^{125}I]iberiotoxin-D19Y/Y36F to guinea-pig urinary bladder membranes. Shown are total (▲), nonspecific (■) and specific (●) binding observed with increasing concentrations of the radioligand. Specific binding was defined by the inclusion of 100 nM unlabeled iberiotoxin. Inset: Scatchard analysis of specific binding data. In this representative plot, the K_D and B_{max} values were 52.8 pM and 130 fmol/mg protein respectively. The mean values are summarized in Section 3.

Table 1
Inhibition of [^{125}I]iberiotoxin-D19Y/Y36F binding by potassium channel ligands in guinea-pig urinary bladder membranes
Inhibition of [^{125}I]iberiotoxin-D19Y/Y36F binding (8 pM) was determined with 7 concentrations of the compounds. The $-\log K_I$ values shown are means of at least three different determinations, each performed in duplicate in the absence or presence of 1 mM EDTA.

Compound	$-\log K_I$	
	Control	+EDTA
[^{125}I]iberiotoxin-D19Y/Y36F	10.34[a]	11.20[a]
Iberiotoxin	10.07	11.36
penitrem A	6.66	8.20
mast cell degranulating peptide	6.54	7.12

[a] Values represent the K_D obtained from saturation experiments.

3.2. Displacement of [^{125}I]iberiotoxin-D19Y / Y36F binding by K^+ channel ligands

Unlabeled iberiotoxin displaced specific [^{125}I]iberiotoxin-D19Y/Y36F binding with a K_I value of 84.8 ± 14.7 pM ($n = 5$; Table 1). In addition to unlabeled iberiotoxin, binding was also inhibited by charybdotoxin, the indole diterpene penitrem A and by mast cell degranulating peptide with K_I values of 2.32 ± 0.99 pM ($n = 5$), 220 ± 131 nM ($n = 3$) and 289 ± 34 nM ($n = 3$), respectively. Other ligands, which have been reported to activate maxi-K^+ channels, *albeit at* high concentrations, including the benzimidazole analog NS1619, the Cl^- channel blocker niflumic acid (Gribkoff et al., 1996), the tertiary carbinol ZD6169 (Hu and Kim, 1997) and (−)-cromakalim (Gelband and McCullough, 1993), did not inhibit [^{125}I]iberiotoxin-D19Y/Y36F binding (up to 100 μM). Blockers of voltage-gated K^+ channels such as margatoxin and α-dendrotoxin (100 nM) or the small conductance K^+ channel blocker apamin (10 μM) were also ineffective at the concentrations tested.

3.3. Modulation of [^{125}I]iberiotoxin-D19Y / Y36F binding by tetraacetic acid analogs

Addition of EDTA in the incubation buffer elicited a concentration-dependent increase in specific [^{125}I]iberiotoxin-D19Y/Y36F binding. As shown in Fig. 2, EDTA enhanced binding with an EC_{50} value of 4.0 ± 1.4 μM ($n = 4$). A maximal increase in binding of about 2.5-fold over control was observed with 100 μM EDTA. Similar increases were also mimicked by EGTA and BAPTA with EC_{50} values of 15.5 ± 3.8 and 11.1 ± 4.0 μM, respectively ($n = 3$).

Although Ca^{2+}-free buffer was employed in all our binding studies, we considered the possibility that these agents could act through removal of membrane-bound

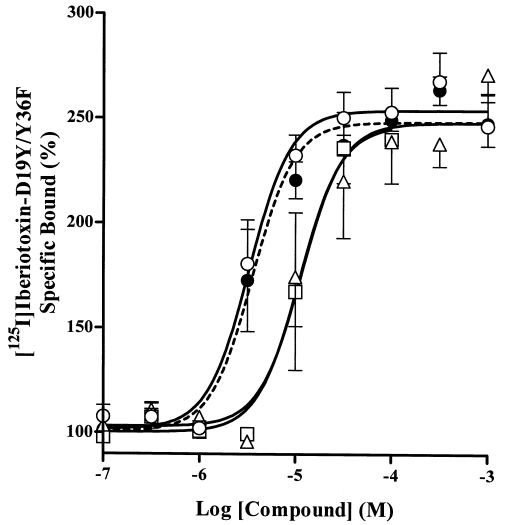

Fig. 2. Modulation of [^{125}I]iberiotoxin-D19Y/Y36F binding to guinea-pig urinary bladder membranes by EDTA and other tetraacetic acid analogs. Membranes were incubated with [^{125}I]iberiotoxin-D19Y/Y36F (8 pM) and varying concentrations of EDTA (○), EGTA (△) or BAPTA (□) as described under Section 2. Shown is the percentage increase with respect to control binding carried out in the absence of compounds (100%). The enhancement of specific binding observed with EDTA was similar when studies were carried out using membranes where residual Ca^{2+} was removed (● dashed line) as described under Section 2. Each data point represents the mean ± S.E. of duplicate determinations from 3 to 6 different experiments.

Ca^{2+}. To investigate this possibility, membranes were pre-incubated with EDTA to remove any membrane-bound Ca^{2+} followed by washout prior to performing the assay. As shown in Fig. 2, the effect of EDTA was still observed in membranes pretreated with EDTA, indicating that the enhancement could not be attributable to the removal of membrane-bound Ca^{2+}, but rather a direct effect on [^{125}I]iberiotoxin-D19Y/Y36F binding.

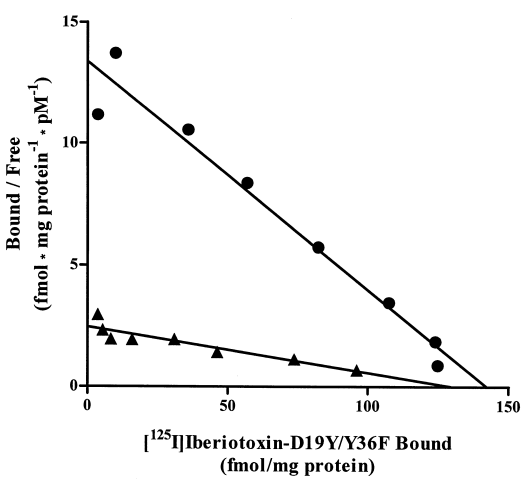

Fig. 3. Modulation of [^{125}I]iberiotoxin-D19Y/Y36F by EDTA. Shown is a representative Scatchard plot of specific [^{125}I]iberiotoxin-D19Y/Y36F binding performed in the absence (▲) and presence (●) of EDTA (1 mM). The K_D values obtained in the absence and presence of EDTA were 52.8 and 10.6 pM, respectively and the B_{max} values were 130 and 142 fmol/mg protein, respectively.

To address the mode of interaction of EDTA, saturation studies with [^{125}I]iberiotoxin-D19Y/Y36F binding were performed in the absence and presence of EDTA. Scatchard analysis of specific binding data showed a significant increase ($P < 0.05$) in the binding affinity ($K_D = 6.3 \pm 1.5$ pM; $n = 9$) but not in binding capacities ($B_{max} = 132 \pm 23$ fmol/mg) in the presence of EDTA compared to the control (Fig. 3). Secondly, when displacement studies were carried out with inhibitors including unlabeled iberiotoxin and penitrem A in the presence of EDTA, an increase in binding affinities were noted, consistent with the results obtained from saturation studies. In addition, as shown in Table 1, the K_I values of unlabeled iberiotoxin, penitrem A and mast cell degranulating peptide (4.41 ± 0.63 pM, 6.3 ± 0.4 nM and 75.2 ± 6.4 nM, respectively, $n = 3–5$) were all higher compared to those values observed in the absence of EDTA.

Additional confirmation was derived from kinetic analysis of [^{125}I]iberiotoxin-D19Y/Y36F to bladder membranes performed in the presence of EDTA. Association

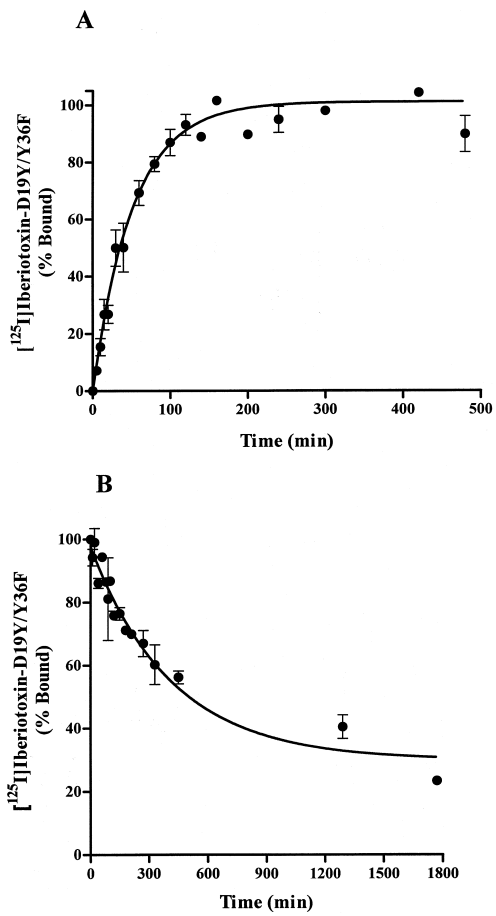

Fig. 4. Time course of association (A) and dissociation (B) of specific [^{125}I]iberiotoxin-D19Y/Y36F binding (20 pM) to bladder membranes in the presence of EDTA (1 mM). Each data point is expressed as % maximal specific bound and represents the mean ± S.E. of duplicate determinations from four different experiments.

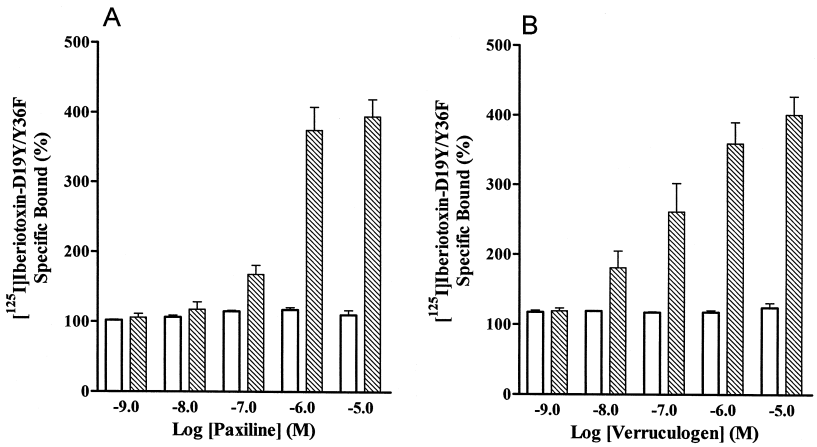

Fig. 5. Modulation of [^{125}I]iberiotoxin-D19Y/Y36F binding to bladder membranes by indole alkaloids. Membranes were incubated with [^{125}I]iberiotoxin-D19Y/Y36F (8 pM) in the presence of EDTA (open bars) and with [^{125}I]iberiotoxin-D19Y/Y36F (2 pM) in the absence of EDTA (filled bars) with different concentrations of paxilline (A) or verruculogen (B) as described in Section 2. The conditions were carefully chosen so as not to deplete the free ligand (maximal bound was 7% of free ligand). Each data point represents the mean ± S.E. of duplicate determinations from 3–4 different experiments.

was monoexponential and reached a plateau at 150 min (Fig. 4A). The association rate constant k_{+1} was 0.000845 ± 0.000053 min^{-1} pM^{-1} ($n = 4$). Dissociation of bound [^{125}I]iberiotoxin-D19Y/Y36F-receptor complex was achieved by addition of a large excess (100 nM) of unlabeled iberiotoxin (Fig. 4B). About 70% dissociation occurred after 1800 min and a single process of dissociation with a k_{-1} value of 0.0024 ± 0.0003 min^{-1} ($n = 4$) was observed. The ratio of k_{-1}/k_{+1}, a measure of the apparent dissociation constant (K_D), showed a value of 4.2 ± 0.6 pM ($n = 4$) that is close to that determined from saturation binding experiments carried out in the presence of EDTA (9.4 pM).

Compounds that have previously been reported to enhance toxin binding to maxi-K^+ channels include the indole alkaloids, paxilline and verruculogen (Hanner et al., 1997; Koschak et al., 1997). As shown in Fig. 5, both paxilline and verruculogen significantly potentiated [^{125}I]iberiotoxin-D19Y/Y36F binding to bladder membranes by about 4-fold (paxilline = 448 ± 14%; verruculogen = 431 ± 11%) with EC_{50} values of 230 ± 15 nM and 101 ± 9 nM respectively ($n = 4$). In contrast, when assays were performed in the presence of EDTA (1 mM) both paxilline and verruculogen did not show an enhancement of [^{125}I]iberiotoxin-D19Y/Y36F binding at any of the concentrations tested (0.3 nM–10 μM).

4. Discussion

This study has characterized the binding properties of [^{125}I]iberiotoxin-D19Y/Y36F to smooth muscle membranes derived from the guinea-pig urinary bladder. Consistent with previous observations (Koschak et al., 1997), this ligand exhibits high affinity and selectivity for maxi-K^+ channels with a very low degree of nonspecific binding, thereby making it possible to study the modulation of maxi-K^+ channels in a manner without interference of voltage gated K^+ channels. The results presented here further demonstrate that tetraacetic acid analogs such as EDTA and EGTA can modulate the binding interactions of [^{125}I]iberiotoxin-D19Y/Y36F.

EDTA evoked a significant 2.5-fold increase in the binding of [^{125}I]iberiotoxin-D19Y/Y36F which could be attributable to an increase of about 4-fold in the ligand binding affinity. The observed enhancement of binding when experiments were performed using membranes prewashed with EDTA indicates that this effect could not be attributable to membrane-bound Ca^{2+}, but rather an action of EDTA on [^{125}I]iberiotoxin-D19Y/Y36F binding itself. This is further supported by the observation that EGTA is about 4-fold weaker in enhancing toxin binding, which is in contrast to the potencies of these agents for Ca^{2+} chelation. Analysis of a variety of competitors showed differences in binding affinities ranging from 0.6–1.5 fold depending on the presence or absence of EDTA (Table 1). These collectively suggest that tetracetic acid analogs may differentially enhance ligand binding via allosteric interactions. Since it is not clear that all the ligands tested are truly competitors and therefore may not bind to exactly the same site, it is not necessarily expected that the interaction by EDTA will affect the affinity of all ligands to the same extent.

Maxi-K^+ channels are widely distributed in a variety of tissues including the brain, chromaffin cells, skeletal muscle T-tubules and smooth muscles and have been shown to be composed of two distinct subunits, α and β (Marty, 1981; Latorre et al., 1989; Garcia-Calvo et al., 1994; Knaus et al., 1995). Previous studies have shown that channels reconstituted from both α and β subunits show faster association and slower dissociation rates for

[^{125}I]charybdotoxin interactions compared to those channels derived from the α subunit alone. Consequently, an increase in binding affinity is observed for the α–β complex compared to the α subunit alone (45 pM vs. 0.8 pM, respectively; Hanner et al., 1997). In our studies, where kinetic parameters for [^{125}I]iberiotoxin-D19Y/Y36F binding were evaluated in the presence of EDTA, a rapid association and a slow dissociation was observed (Fig. 3). Although the effect of EDTA on the kinetic parameters of [^{125}I]iberiotoxin-D19Y/Y36F has not been directly compared with similar experiments in the absence of EDTA, the K_D value calculated from kinetic parameters in the presence of EDTA (4.2 pM) was close to that derived from saturation experiments in the presence of EDTA (6.3 pM), but significantly different from the values obtained in saturation experiments performed in the absence of EDTA (45.6 pM). Therefore, the increase in [^{125}I]iberiotoxin-D19Y/Y36F binding observed in the presence of EDTA could be explained by an increase in toxin affinity.

The potencies for the displacement of [^{125}I]iberiotoxin-D19Y/Y36F binding by iberiotoxin and penitrem A are consistent with those previously reported in tracheal smooth muscle membranes (Koschak et al., 1997). Although mast cell degranulating peptide was found to inhibit binding with a K_I value of 289 nM, this value is at least 1000-fold higher than required for displacement of binding to voltage-gated potassium channels in rat synaptic brain membranes (Taylor et al., 1984; Giangiacomo et al., 1995). Moreover, α-dendrotoxin and margatoxin did not displace binding even at the highest concentration tested. On the other hand, peptide blockers of small-conductance K^+ channels such as apamin, K_{ATP} channel activators such as (−)-cromakalim or ligands reported to activate maxi-K^+ channels *albeit* at high concentrations such as niflumic acid, NS1619 and ZD6169 failed to displace binding at the maximal concentrations evaluated.

Indole alkaloids, paxilline and verruculogen, are potent blockers of smooth muscle maxi-K^+ channels and have been shown to increase spontaneous contractility in smooth muscles including those from the urinary bladder (Knaus et al., 1994; Gribkoff et al., 1996; DeFarias et al., 1996). These compounds have been shown to enhance [^{125}I]charybdotoxin binding to maxi-K^+ channels (Knaus et al., 1994). More recently, it has been shown that paxilline enhances [^{125}I]charybdotoxin binding to membranes of cells transfected with the α subunit alone or with the α–β complex, suggesting that the interaction of ligands like paxilline are to the α subunit and its action is independent on the presence of the β subunit (Hanner et al., 1997). In our studies, paxilline and verruculogen both increased [^{125}I]iberiotoxin-D19Y/Y36F binding about 440% above control values when assays were performed in the absence of EDTA (EC_{50}, 230 nM and 100 nM, respectively). Previous studies have shown that the modulation of [^{125}I]charybdotoxin binding by these compounds is due to an increase in the toxin affinity, perhaps by a positive allosteric interaction (Knaus et al., 1994) similar to those described in our present studies with tetraacetic acid derivatives. The enhancement of [^{125}I]iberiotoxin-D19Y/Y36F binding by indole alkaloids is significantly higher (440%) than those evoked by EDTA or EGTA (250%). Further, this potentiation of binding by paxilline and verruculogen was not observed in the presence of EDTA. Since the effects of indole alkaloids and tetraacetic acids are not additive, the conformational changes may be of similar nature. However, additional studies will be necessary to examine the mechanisms in further detail.

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