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Identification and Molecular Characterization of the Isoquinoline Rat Intestinal Binding Site Using 6,7-Dimethoxy-4-(4′-amino-3′-[^{125}I]iodobenzyl) Isoquinoline

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SUMMARY

The mechanism of the relaxant action of isoquinolines on smooth muscle is conjectural. In order to gain further insight into the intestinal action of isoquinolines, we have synthesized an isoquinoline derivative which can be radioiodinated, resulting in the obtention of a ligand with a high specific activity. 6,7-Dimethoxy-4-(4′-aminobenzyl) isoquinoline (DMABI) is an arylamine analogue of the most relaxating isoquinoline derivative, i.e., 6,7-dimethoxy-4-(4′-chlorobenzyl) isoquinoline. Its iodinated derivative, 6,7-dimethoxy-4-(4′-amino-3′-[^{125}I]iodobenzyl) isoquinoline (^{125}I-DMABI) binds reversibly to rat intestinal membranes. Binding is rapid, saturable, and temperature dependent. The binding of ^{125}I-DMABI to intestinal membranes is competitively inhibited by identical concentrations of unlabeled DMABI or iodo-DMABI in the range between 10^{-8} and 10^{-5} M. Scatchard analysis indicates the existence of two classes of binding sites: a class with a low capacity (14±2 pmol/mg of protein) and a K_d = 0.10±0.02 μM, and a class with a high capacity (240±31 pmol/mg of protein) and a K_d = 8.0±1.1 μM. Specific binding of the radioiodinated ligand is inhibited by a variety of 4-benzyl isoquinolines and 1-benzyl isoquinolines. Structure-activity relationship demonstrates the primordial role of C-6 and C-7 methoxy groups and the important role of 4-benzyl on configuration related to the isoquinoline nucleus. A high significant correlation between competitive binding (K_i) and relaxant effect in rat intestine (IC_{50}) is observed and strongly suggests that the isoquinoline-binding site mediates the pharmacologic response. Upon photolysis, this ligand incorporates irreversibly into rat intestinal membranes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography reveal a major ^{125}I-DMABI-labeled protein with molecular weight of 36,000 and two minor proteins with molecular weights of 52,000 and 26,000. The labeling of these proteins is specific since it is completely abolished by 100 μM DMABI. Scanning of autoradiographs and integration of peaks show that the probe binds with the same apparent affinity to the three proteins. These findings indicate the utility of this novel high affinity radioiodinated probe as a tool for elucidating the mechanism of action of isoquinoline.

Papaverine [6,7-dimethoxy-1-(3′,4′-dimethoxybenzyl isoquinoline] is the most well investigated isoquinoleic compound exhibiting relaxant activity in the vascular and the intestinal smooth muscles. However, the mechanism of the relaxant effect of isoquinoline is conjectural. Initial works suggested that isoquinoline acts by inhibiting cAMP-PDE in consonance with the relaxant action of cAMP on smooth muscle (1–6). Alternatively, since calcium is now assumed to play a pivotal role in smooth muscle relaxation, several authors investigated the possible effect of papaverine on Ca^{2+} movements (6–12). As suggested, this effect may be subsequent to cAMP-PDE inhibition and related to cAMP-induced Ca^{2+} efflux from intracellular stores and/or Ca^{2+} influx from extracellular medium (13, 14). More recently, Kimura *et al.* (15) indicated that papaverine alters Ca^{2+} fluxes in the hog biliary muscle, through a cAMP-independent pathway. Moreover, using [^{3}H]papaverine as ligand, these authors suggested that the action of papaverine involves its interaction with a protein located in the plasma membrane.

In order to gain further insight into the site of action of papaverine and papaverine-like compounds in intestine, we have developed a radioiodinated isoquinoline derivative, ^{125}I-DMABI. This compound is a derivative of PV-2, a 4-benzyl isoquinoline with intestinal and vascular spasmolytic activity

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ABBREVIATIONS: cAMP-PDE, cyclic AMP phosphodiesterase; DMABI, 6-7-dimethoxy-4-(4′-aminobenzyl) isoquinoline; DMAIBI, 6,7-dimethoxy-4-(4′-amino-3′-iodobenzyl) isoquinoline (iodo-DMABI); ^{125}I-DMABI, 6,7-dimethoxy 4-(4′-amino 3′-[^{125}I]iodobenzyl isoquinoline); PV-2, 6,7-dimethoxy-4-(4′-chlorobenzyl isoquinoline); TLCK, tosyl-L-lysine-chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; D-600, gallopamil; PK 11195, 1-(2-chlorophenyl-*N*-methyl-*N*-(1-methyl-2-propenyl)-3-isoquinoline carboxamide; Be 2254 or HEAT, DL-2-[β-(4-hydroxyphenyl) ethyl aminoethyl] tetralone; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

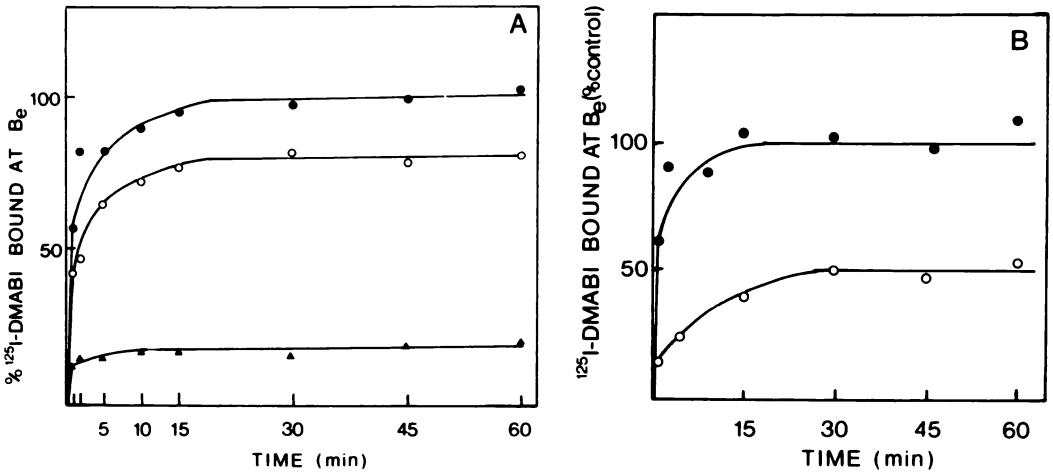


Fig. 1. A. Kinetics of ^{125}I-DMABI binding. The figure is representative of an experiment performed at 30°, pH 7.5, in Tris-HCl buffer (50 mM, with 5 mM $MgCl_2$/100 mM NaCl/protease inhibitors). At the indicated times, aliquots were withdrawn and association was terminated by filtration. Specific binding was defined by 10^{-4} M unlabeled DMABI. Association was initiated by adding intestinal membranes (40 μg of proteins) to incubation flasks containing ^{125}I-DMABI (0.15 μCi, 0.75 nM). ●, total binding; ▲, nonspecific binding; ○, specific binding. The data shown are from three separate experiments each performed in duplicate. B_e, equilibrium binding. B. Time course for association on specific ^{125}I-DMABI binding to rat intestinal membranes at 15° (●) and 30° (○). The data shown are from two separate experiments each performed in triplicate.

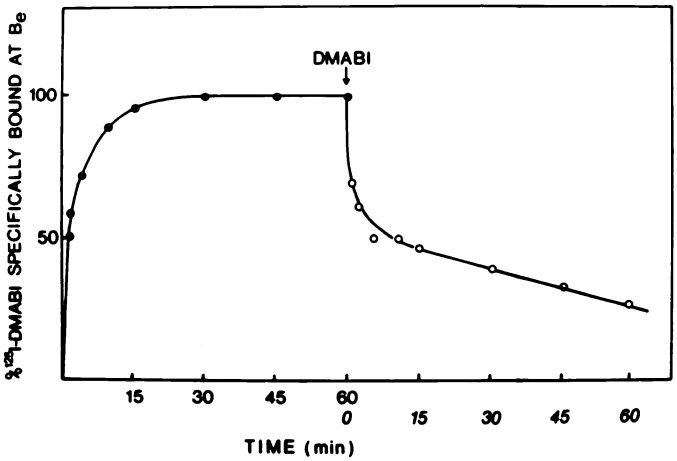


Fig. 2. Kinetics of formation and dissociation of the ^{125}I-DMABI-rat intestinal membrane complex. The figure is representative of experiments performed at 30°, pH 7.5. Specific binding as defined by 10^{-4} M unlabeled DMABI is plotted. Nonspecific binding represents 20% of total binding at equilibrium and is already substracted from the figure. As observed, the association reaction (●) had a half-life of 7 min. After equilibrium had been reached (60 min) and checked to be stable for more than 60 min, the rate of dissociation of the complex (○) was monitored following addition of 10^{-4} M unlabeled DMABI. The dissociation reaction had a half-life of 42 min. The figure demonstrates the reversibility and saturability of ^{125}I-DMABI binding to rat intestinal membranes. B_e, equilibrium binding; and B_t (not shown), binding at a given time, t.

higher than that observed with papaverine (16, 17). The replacement of the 4′-chloro by 4′-amino substituent does not alter pharmacological activity and makes it possible to prepare a radiolabeled derivative of high specific activity by iodination (18).

In this report, we identify in rat intestinal membrane a high affinity binding site of ^{125}I-DMABI which displays a structural requirement compatible with the pharmacology of the relaxant effect of isoquinolines. Moreover, we identify by photolabeling 52,000, 36,000, and 26,000 molecular weight proteins as isoquinoline-binding sites.

Materials and Methods

Carrier-free Na^{125}I (IMS-300, 14.7 mCi/μg of iodine) was purchased from Amersham (Buckinghamshire, England). Rats (Sprague-Dawley) were obtained from Charles River (St. Aubin-les-Elbeuf, France).

The following drugs or chemicals were obtained as follows: PMSF, bacitracin, leupeptin, TLCK, GTP, ATP, AMP, adenosine, dichloroadenosine, papaverine, 3-quinuclidinyl benzoate, atropine, acetylcholine, isobutylmethylxanthine, and chlorpromazine from Sigma Chemical Co.; theophylline and cAMP from Calbiochem; *N*-ethylmaleimide from BDH Chemicals Ltd.; BSA, fraction V from Miles Laboratories; and tetrahydropapaveroline, Aldrich.

The following drugs were generously donated by the companies listed:

Isoproterenol, Winthrop; propanolol, ICI Pharmaceuticals; prasozin, Pfizer; Be 2254 or HEAT, Beiersdorf AG: verapamil and D-600, Knoll AG; nifedipine (Bayer Pharmaceuticals), prenylamine (Hoechst), diltiazem (Synthelabo), trifluoperazine, SKF Laboratories; diazepam, flunitrazepam, and Ro 20-1724, Roche Products; PK 11195, Pharmuka Rhone Poulenc Industries; cinnarizine, Delalande; and bepredil, Riom Laboratories. Other biochemicals were from Sigma. Chemicals were usually from Merck and all solvents and reagents were of the highest grade available.

Isoquinoline derivatives were synthetized by H. Christinaki and C. Viel and were characterized for purity and chemical composition by ^{1}H-NMR, IR, and elemental analysis.

Contractile studies. Male Sprague-Dawley rats (250–300 g) were decapitated and their duodedums were removed. The duodenum (approximately 2 cm) was mounted in an organ bath in 10 ml of Krebs-Ringer bicarbonate buffer gassed with 95% O_2/5% CO_2, pH 7.5, and containing: NaCl, 118.5 mM; KCl, 4.74 mM; $MgSO_4$, 1.18 mM; KH_2PO_4, 1.18 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 24.9 mM; and glucose, 10 mM. Resting tension of 1 G force was maintained throughout the experiment. Tissues were allowed to equilibrate for 2 hr before addition of any drug.

Contractile responses were elicited by 1 mM $BaCl_2$. Relaxation of duodenum by isoquinolines was tested by their ability to cause relaxation of $BaCl_2$-contracted strips. Isoquinolines were dissolved in DMSO, and relaxation in response to DMSO was subtracted from the response to each agent. Concentrations of the agents that cause 50% relaxation of the $BaCl_2$-induced contraction (IC_{50}) were determined from concen-

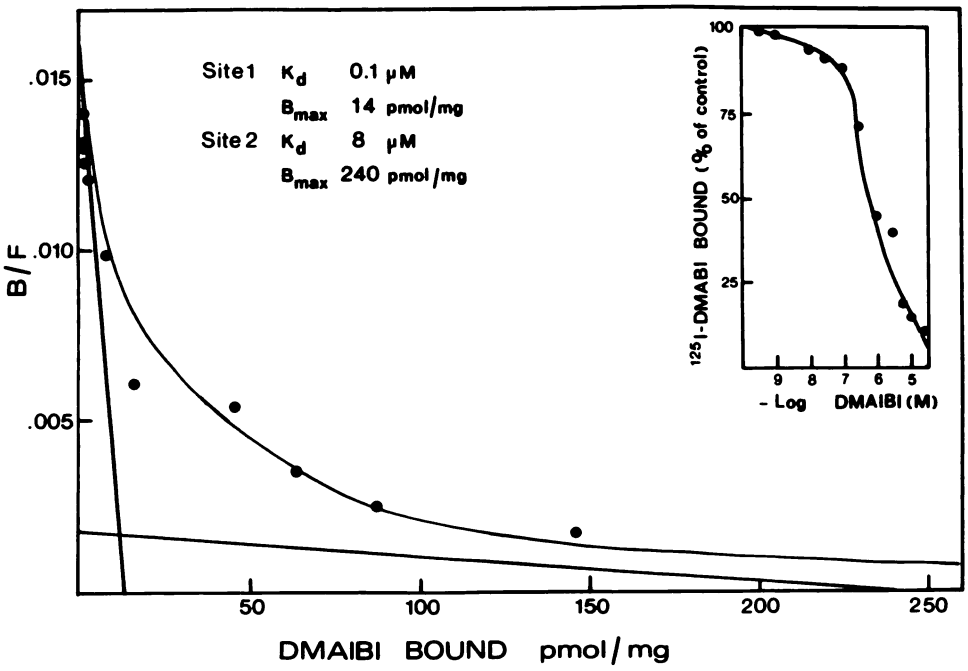


Fig. 3. Scatchard analysis of DMAIBI binding to rat intestinal membranes. Binding of ^{125}I-DMABI to rat intestinal membranes (50 μg of protein) was measured under equilibrium conditions, for 60 min at 30° and pH 7.5 in the presence of concentrations of unlabeled iodo-DMABI (DMAIBI) in the range 10^{-8}–10^{-5} M. Nonspecific binding was defined at 10^{-5} M DMAIBI. Mean specific binding values (●) ± standard errors were determined from four equilibrium experiments, with each experiment done in duplicate. The *curve* represents the best mass action fit to the data. *Inset*, competitive inhibition of ^{125}I-DMABI binding to rat intestinal membranes by increasing concentrations of unlabeled DMAIBI.

TABLE 1

Biochemical and biophysical properties of isoquinoline binding site as revealed by ^{125}I-DMABI

Conditions	Binding of [^{125}I]-DMABI (fraction of control)
1. Heat treatment	
15 min at 55°	1.00
15 min at 65°	0.90
15 min at 100°	0.60
2. Freeze-thawing	1.01
3. Enzymes	
Trypsin (20 μg/ml) 60 min at +30° followed by trypsin inhibitor (TLCK, 40 μg/ml)	0.09
Trypsin (6 μg/ml) 18 hr at +4° followed by trypsin inhibitor (TLCK, 20 μg/ml)	0.67
4. Alkylating and reducing agents	
N-Ethylmaleimide (1 mM)	1.07
Iodoacetamide (1 mM)	1.00
Mercaptoethanol (1 M)	1.02
5. Detergent	
Triton (1%) 60 min at +30°	0.06
6. Chelators	
EGTA IC_{50} (μM)	0.2
7. Pretreatments by salts	
NaCl (400 mM)	1.03
KCl (400 mM)	0.50
$CaCl_2$ (0.01 mM)	1.02
$MgCl_2$ (100 mM)	1.01
8. Nucleotides	
AMP (1 mM)	1.00
cAMP (1 mM)	1.02
ATP (1 mM)	1.00
GTP (1 mM)	1.01

tration-response curves obtained by averaging the responses of three to five experiments.

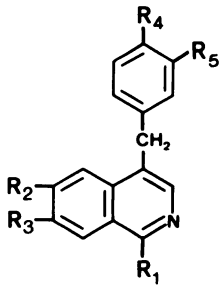
Preparation of ^{125}I-DMABI. DMABI was radiolabeled with ^{125}I by the chloramine T method as previously described (18). Briefly, the following were added sequentially at room temperature to a glass tube; 6 μl of DMABI in DMSO (1 mg/ml), 24 μl of 1 M sodium acetate buffer, pH 5.6, 2 μl (2 mCi) of ^{125}I solution, and 6 μl of chloramine T (1 mg/ml of H_2O). After 1 min incubation, the reaction was terminated by adding 8 μl of sodium metabisulfite (1 mg/ml in acetate buffer, pH 5.6) and 5 μl of 1 N NaOH. ^{125}I-DMABI and DMABI unreacted were extracted by ethyl acetate (four times in 0.5 ml). After concentration under N_2 pressure, ^{125}I-DMABI was separated from unlabeled DMABI on a Sephadex LH-20 column (0.9×30 cm) eluted with Et(OH), CH_3COOH, 0.1%. Due to the complete separation of ^{125}I-DMABI and unlabeled DMABI, a high specific activity was obtained (2175 Ci/mmol). Fractions corresponding to the peak of ^{125}I-DMABI on the Sephadex LH-20 column were pooled and stored frozen at −20. ^{125}I-DMABI is radiochemically pure for at least 2 months. The specific activity of the radiolabeled DMABI after storage is calculated from the half-life of ^{125}I. For binding assays the specific activity was adjusted at 980 Ci/mmol by isotopic dilution with unlabeled iodo-DMABI (DMAIBI).

Preparation of intestinal membranes from radioligand binding. Male Sprague-Dawley rats (200–250 g) were killed by decapitation and the small intestine from each animal was removed rapidly and washed with ice-cold buffer (Tris-HCl, 50 mM; $MgCl_2$, 5 mM; NaCl, 100 mM; EDTA, 1 mM; bacitracin, 100 μg/ml; TLCK, 10 μg/ml; PMSF, 100 μM; leupeptin, 10 μg/ml at pH 7.5). The tissue was minced and homogenized using a Polytron homogenizer in the same buffer, to a final concentration of 100 mg of original wet tissue weight/ml of buffer. The homogenate was filtered through four layers of cheese cloth. The tissue homogenate was washed five times by centrifugation at 35,000 × *g* for 10 min at +4° (Sorvall RC5-B, rotor SS-34) followed by suspension of the pellet in fresh buffer. The last suspension was rehomogenized with five strokes in a glass-glass homogenizer. The final pellets were resuspended to a concentration of 100 mg of original wet tissue weight/ml of buffer and frozen at −80° until use. It will be referred to as crude intestinal membranes (19).

Radioligand binding assays. Prior to assay, the crude intestinal membranes were thawed and diluted 10-fold in the above-mentioned Tris-HCl buffer without EDTA and washed two times. The resuspension was conducted with a glass-glass homogenizer. Assays were performed in polypropylene tubes in a total volume of 0.2 ml and incubated at 30°. Assays were initiated by the addition of 100 μl of crude membranes (30–60 μg of protein) to a mixture of 50 μl of ^{125}I-DMABI (0.15 μCi, 900 Ci/mmol, 0.75 nM) and 50 μl of buffer or competing agent. Incubations were terminated by a 5-fold dilution with ice-cold buffer (50 mM Tris-HCl, 100 mM NaCl, 0.2% BSA, pH 7.5) and rapid filtration on Whatman GF/C filters. The trapped membranes were washed two times with 12 ml of the above BSA-containing buffer. The filters were

TABLE 2

Structures of 4-benzyl isoquinolines, their competition for the binding of ^{125}I-DMABI to the rat crude intestinal membranes, and their relaxant effect on rat duodenum



Compound	Binding assay[a]						Relaxant effect[b]		
	R_1	R_2	R_3	R_4	R_5	K_i[a]	Potency	IC_{50}	Potency
						μM		μM	
DMABI	H	OCH_3	OCH_3	NH_2	H	0.8 ± 0.1	100	4.0 ± 0.8[c]	100
PV-2	H	OCH_3	OCH_3	Cl	H	1.5 ± 0.3	53	7.0 ± 0.9	57
3′-OH PV2, 1a	H	OCH_3	OCH_3	Cl	OH	3.0 ± 0.8	27	8.0 ± 1.2	50
6-OH-PV2, 1b	H	OH	OCH_3	Cl	H	4.0 ± 0.5	20	10.0 ± 1.9	40
7-OH PV-2, 1c	H	OCH_3	OH	Cl	H	15.0 ± 1.6	5.3	40.0 ± 5.1	10
6,7-OH PV-2, 1d	H	OH	OH	Cl	H	>1000		>1000	
Compound 2a	H	OCH_3	OCH_3	OH	H	100 ± 1.5	0.8	39 ± 4.5	10
Compound 2b	H	OCH_3	OCH_3	NO_2	H	4.0 ± 0.7	20	8 ± 1.0	50
Compound 2c	H	OCH_3	OCH_3	N_3	H	1.0 ± 0.1	80		50
Compound 2d	H	OH	OH	OH	H	>1000		>1000	
Compound 2e	H	OH	OH	NH_2	H	>1000		>1000	
Compound 2f	H	OCH_3	OCH_3	$N(CH_3)_2$	H	7.0 ± 2.1	11.4	27[d]	15
Compound 2g	CH_3	OCH_3	OCH_3	Cl	H	300 ± 42	0.27	91 ± 12	4.4
Compound 2h	H	OH	OH	NO_2	H	>1000		>1000	
Compound 2i	H	OH	OH	$N(CH_3)_2$	H	>1000		>1000	
Compound 1e, ketone of PV2	H	OCH_3	OCH_3	Cl	H	2.0 ± 0.5	40	7.0 ± 2.1	57
Compound 1f (N-O)	H	OCH_3	OCH_3	Cl	H	300 ± 27	0.27	150 ± 27	2.6
Compound 2j	H	OCH_3	OCH_3	OCH_3	OCH_3	15 ± 2.2	5.3	18[d]	22
Compound 2k	H	OCH_3	OCH_3	OCH_3	H	0.09 ± 0.03	888	44[d]	9
6,7-Dimethoxy isoquinoline		OCH_3	OCH_3			1000 ± 110	0.8		

[a] Values are from three separate experiments performed in duplicate.
[b] Values are from three or four separate experiments.
[c] Refs. 16, 17, and 28.
[d] Ref. 17.

then counted for radioactivity in a Packard Auto-Gamma 800 gamma counter at a counting efficiency of 60%. Due to the rather hydrophobic nature of the ligand, the nonspecific binding was significantly lowered when a low oncentration of BSA was included in the washing buffer used during the filtration. Specific binding was calculated as the difference between the amount of ^{125}I-DMABI bound in the absence (total binding) and presence (nonsaturable binding) of 100 μM unlabeled iodo-DMABI (DMAIBI). Under optimal conditions, the specific binding of ^{125}I-DMABI is approximately 80% of the total binding. We have verified that specific ^{125}I-DMABI binding was proportional to the crude membrane concentration up to 225 μg of protein/ml. Each binding measurement was performed in duplicate.

The constants, K_i, for the inhibition of ^{125}I-DMABI binding by isoquinolines were defined as the concentrations of isoquinoline derivatives eliciting half-maximal inhibition of ^{125}I-DMABI. The binding potency relative to that of DMABI was then calculated for each analog as (K_i DMABI/K_i A)×100, where K_i DMABI and K_i A are the inhibition constants of unlabeled DMABI and analog, respectively. The statistical significance of the difference between K_i values was assessed by the Student's t test. Measurement of proteins was determined by the method of Bradford (20) using BSA as standard.

Radioligand stability. The stability of the ^{125}I-DMABI during the incubation with intestinal membranes was tested in the supernatant of incubation. Aliquots of incubations spotted in Whatman LK6DF were developed in solvent systems chloroform/methanol/acetic acid, 75:20:5 by volume. The chromatogram was exposed to a Kodak Industrex AX 4 film for 4 days at room temperature.

Photoaffinity labeling of intestinal crude membranes with ^{125}I-DMABI. Crude intestinal membranes (600 μg of protein) were incubated in Sorvall SS 34 polycarbonate tubes in a total volume of 2 ml of buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, 100 μg/ml of bacitracine, 10 μg/ml of TLCK, 100 μg/ml of PMSF, 10 μg/ml of leupeptin, pH 7.5), including approximately 5–10 nM radioactive ligand and either buffer or competing agent (DMABI, 0.1–100 μM). This protocol did not alter any of the ligand-binding characteristics. Incubation was carried out for 60 min at 30°. The incubation mixture was then diluted to 30 ml with buffer, including 0.5% BSA, and centrifuged 10 min at 40,000× g. The membranes were then homogenized in the BSA-containing buffer and recentrifuged for the same length of time at 40,000× g. The supernatant was removed, and the pellet was immediately resuspended in 1 ml of Tris-HCl buffer without BSA. Membranes containing bound ^{125}I-DMABI were photolabeled by exposure to a 254-nm UV lamp (model R-52G, UVP Inc., CA) in a Petri dish for 15 min. The photolabeled material was centrifuged at 20,000× g for 15 min, and the resulting pellets were resuspended by five successive passages through a 25 gauge needle in 60 mM Tris buffer, pH 6.8, containing 10% (w/v) glycerol, 0.001% (w/v) bromophenol blue, and 3% (w/v) sodium dodecyl sulfate. After heating at 60° for 30 min, the suspension was centrifuged at 40,000 × g for 15 min, and aliquots of supernatant were applied to 5–20% polyacrylamide slab gel (1.5 mm thick) according to the Laemmli method (21) with a 3% stacking gel.

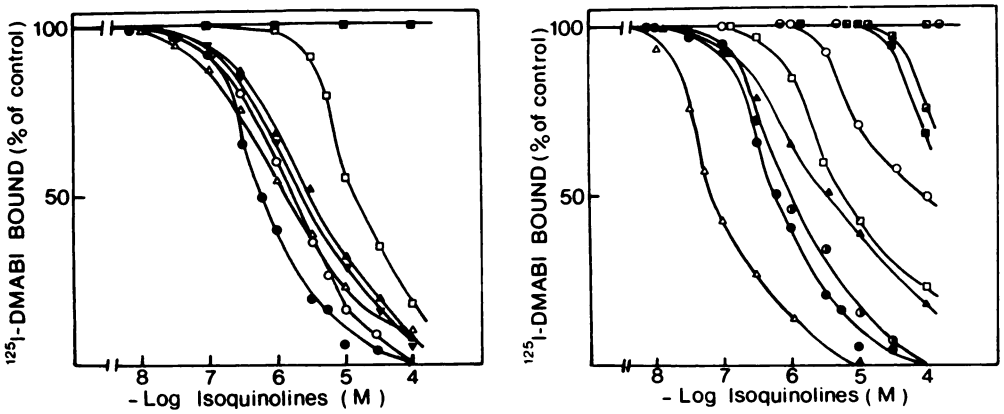


Fig. 4. Competition of ^{125}I-DMABI with 4-benzyl isoquinolines in rat intestinal membranes. A constant concentration of ^{125}I-DMABI (0.75 nM) was incubated with increasing concentrations of 4-benzyl isoquinolines and the assays were performed for 60 min, at 30° and pH 7.5 *^{125}I-DMABI BOUND* refers to specific binding of ^{125}I-DMABI to intestinal membranes (30 μg of protein/0.2 ml of assay) as determined in the presence of 10^{-4} M unlabeled DMABI. The *line* through the experimental *points* represents the best fit to the data of three or four experiments performed in duplicate. For the sake of clarity, standard errors are not indicated. They were always below 15% of mean values. *Left:* ●, DMABI; △, PV-2; ▼, compound 1a (3′-OH PV-2), ▲, compound 1b (6-OH PV-2). □, compound 1c (7-OH PV-2); ■, compound 1d (6,7-OH PV-2), ○ compound 1e (ketone of PV-2). *Right:* ●, DMABI; ○, compound 2a (4′-OH); ▲, compound 2b (4′-NO_2); ◑, compound 2c (4′-N_3), incubated in the dark; ⊖, compound 2d (6,7,4′-OH); ◪, compound 2e (6,7-OH DMABI); □, compound 2f [4′-N(CH_3)$_2$]; ◪, compound 2g (1-CH_3 DMABI); △, compound 2j (4′-OCH_3); ■, compound 1f (N-oxide PV-2). For structures see Table 2.

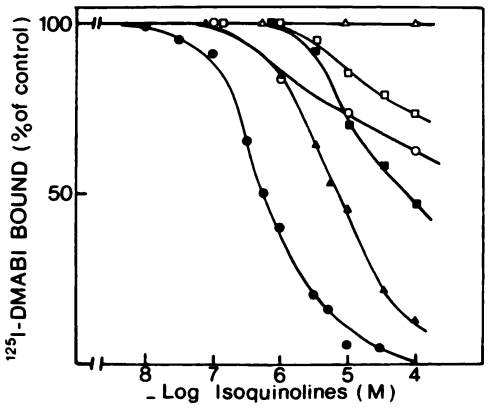


Fig. 5. Competition of ^{125}I-DMABI with 1-benzyl isoquinolines in rat intestinal membranes. Experimental conditions are described in the legend to Fig. 4. ●, DMABI; ▲, papaverine; ■, ethaverine; □, drotaverine; ○, 6,7-dimethoxy isoquinoline; △, tetrahydropapaveroline.

After electrophoresis for 16 hr, the gels were dried and exposed for 3–8 days at −80° to a Trimax type XM film (3M Co., St. Paul, MN) with a Trimax intensifying screen. Autoradiographs were scanned at 600 nm using a Joyce microdensitometer (MKIIIC).

Results

General properties of ^{125}I-DMABI binding. Specific binding was time and temperature dependent. At 30°, steady state binding was reached at 15 min and was maintained until at least 60 min (Fig. 1A). When the temperature of incubation was decreased to 15°, the rate of association was slower and the amount of specific ^{125}I-DMABI binding represented 50% of that observed at 30° (Fig. 1B). At both temperatures, specific binding represented 80% of total binding. As tested by thin layer chromatographic analysis, there was no alteration of ^{125}I-DMABI during incubation with crude intestinal membranes. ^{125}I-DMABI-binding site complexes were almost completely reversible at 30° since 75% of complexes were dissociated after 60 min (Fig. 2). The concentration dependence of DMABI binding was studied at equilibrium by adding increasing concentrations of unlabeled iodo-DMABI (DMAIBI) to a fixed concentration of ^{125}I-DMABI (0.75 nM). It appears that unlabeled iodo-DMABI in the concentration range between 10^{-8} and 10^{-5} M competes with ^{125}I-DMABI for binding to crude intestinal membranes (Fig. 3, *inset*). Scatchard analysis of the data (Fig. 3) gave a curve with an upward concavity, suggesting that ^{125}I-DMABI interacts with two populations of binding sites: a population with a low capacity (14±2 pmol/mg of protein) and a K_d = 0.10±0.02 μM, and a population with a high capacity (240±31 pmol/mg of protein) and a K_d = 8.0±1.1 μM (mean ± SE of four experiments).

The dependence of ^{125}I-DMABI binding to crude intestinal membranes on trypsin digestion, heat, and ionic treatments and other chemical treatments was investigated. Table 1 indicates that ^{125}I-DMABI binding is sensitive to proteolytic degradation, suggesting the proteic nature of binding sites. ^{125}I-DMABI binding was relatively heat sensitive. Pretreatment of crude membranes by ionic treatments (NaCl, $MgCl_2$, and $CaCl_2$) did not modify ^{125}I-DMABI binding. By contrast, KCl pretreatment (400 mM) decreased the ^{125}I-DMABI binding. The total solubilization of the binding site was obtained by 1% Triton X-100 (Table 1). Alkylating and reducing agents did not alter the binding of ^{125}I-DMABI, indicating that neither an SH group nor a disulfide bridge is essential for the interaction of the ^{125}I-DMABI with its binding site. EGTA drastically reduced the ^{125}I-DMABI binding (IC_{50} 2×10^{-7} M).

Pharmacological profile of ^{125}I-DMABI binding. The structural requirement and pharmacological profile of the ^{125}I-

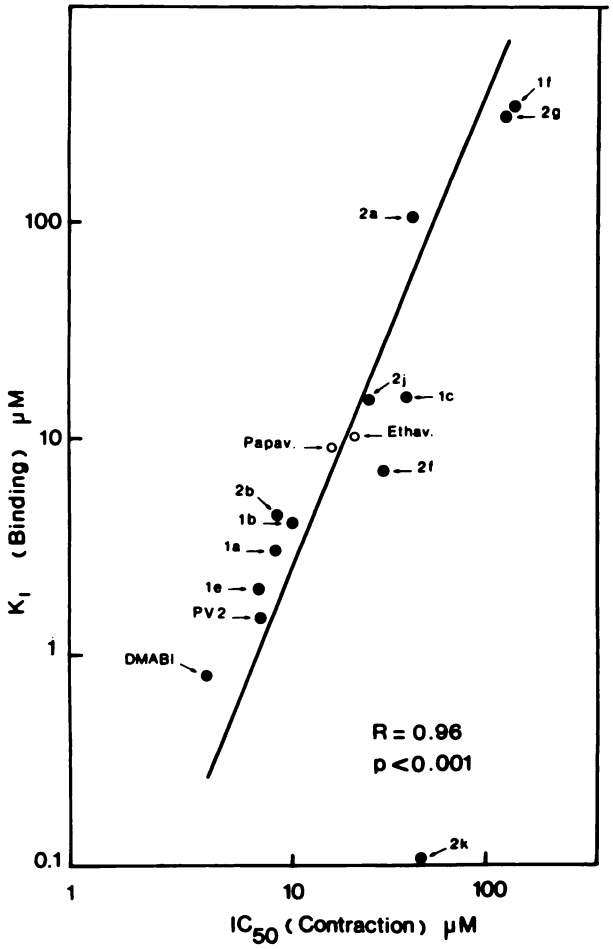


Fig. 6. Correlation between the binding affinity of DMABI and 4-benzyl isoquinoline derivatives (K_i) and the inhibition of $BaCl_2$ contraction (IC_{50}) by DMABI and 4-benzyl isoquinolines. K_i, IC_{50}, and number affected to each point refer to compounds listed in Table 2.

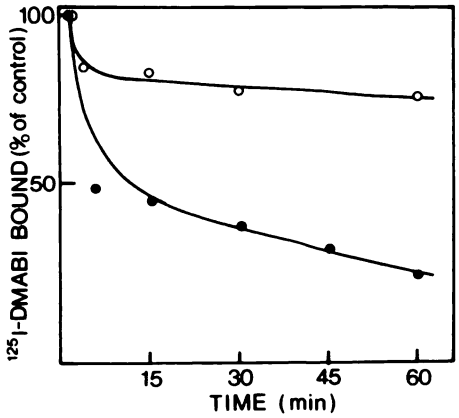


Fig. 7. Inhibition of ^{125}I-DMABI dissociation from rat intestinal membranes due to UV cross-linking. After association for 60 min at 30° and pH 7.5, the dissociation was monitored by adding 10^{-4} M unlabeled DMABI, with (○) and without (●) prior UV photolysis (254 nm). Each *point* is the mean of two experiments each performed in triplicate. Standard errors are 10% of mean values.

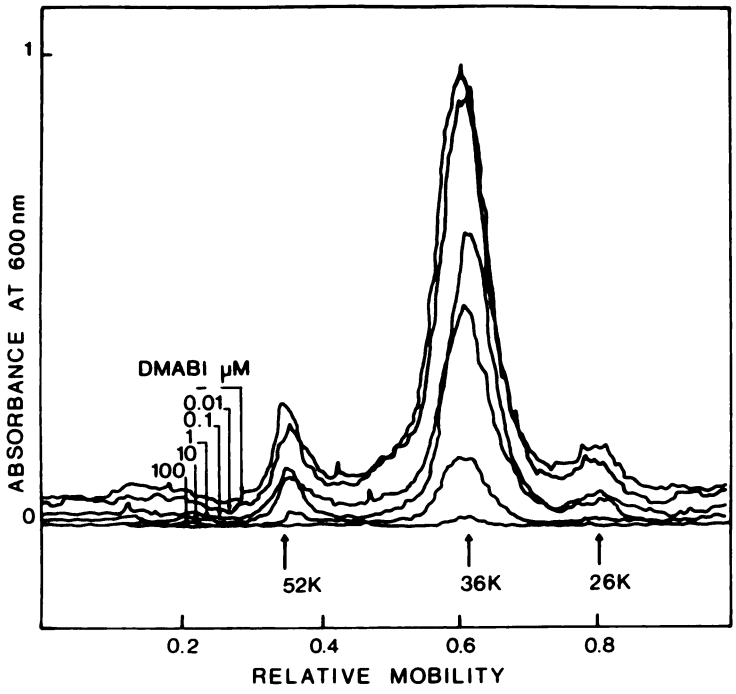
DMABI-binding site were tested with several isoquinoline derivatives and various drugs (Table 2, Figs. 4 and 5). Most active derivatives display inhibition curves parallel to that obtained with unlabeled DMAIBI. It appears that: 1) when the methoxyl groups in positions R_2 and R_3 (6,7-isoquinoline) are substituted by hydroxyl groups, the resulting compounds do not recognize at all the DMABI-binding sites (6,7-OH PV-2, compounds 2d, 2e, 2h, 2i, and tetrahydropapaveroline). In contrast, when only one of the two methoxyl groups is modified, the resulting compounds (6-OH PV-2 and 7-OH PV-2) retain some affinity for the binding site, i.e., 5–20-fold lower than DMABI. 2) When the methyl group is present in the C-1 position of the isoquinoline moiety, an important decrease of affinity is observed; likewise, when the N^2 position of the isoquinoline moiety is *N*-oxided, a similar decrease in affinity occurs (compounds 2g and 1f). 3) When the amine in the R_4 position is replaced by Cl (PV-2), NO_2 (compound 2b), N_3 (compound 2c), or $N(CH_3)_2$ (compound 2f), the affinity is decreased by less than 10 times. In contrast, when hydroxyl is present in the R_4 position, the affinity decreases (compound 2a). 4) Replacement of amine by methoxyl enhances the affinity (compound 1k). In contrast, the replacement by methoxyl in 3′ and 4′ results in a 20-fold decrease of the affinity (compound 2j). Similarly, when the 6,7-dimethoxy-isoquinoline is not benzylated in position 4, the affinity is 100 times lower than in DMABI (Fig. 5).

1-Benzyl isoquinolines, i.e., papaverine (IC_{50} = 8×10^{-6} M) and ethaverine (IC_{50} = 10^{-6} M), also compete with ^{125}I-DMABI for binding. This interaction is related to a structural similarity with DMABI rather than to the capacity of these drugs to inhibit cAMP-PDE. Indeed, other phosphodiesterase inhibitors such as xanthine derivatives, e.g., isobutylmethylxanthine and theophylline, and others, e.g., Ro 20-1724, cannot displace ^{125}I-DMABI. These results suggest that ^{125}I-DMABI does not label the catalytic site of cAMP-PDE. This is further indicated by the absence of competition observed with cAMP. Similar absence of effect is observed with various nucleotides (Table 1).

The important structural specificity of the DMABI intestinal binding site for the isoquinoline series is further demonstrated by the absence or very low displacement observed with various drugs used in concentrations up to 10^{-5} M, e.g., α-adrenergic agonists (epinephrine) and antagonists (prazosin and Be 2254), β-adrenergic agonists (isoproterenol) and antagonists (propanolol), central (diazepam and flunidiazepam) and peripheral (PK 11195) benzodiazepines, muscarinic and nicotinic drugs (acetylcholine and quinuclidinylbenzilate), so-called "calmodulin antagonists" (chlorpromazine and trifluoperazine), and adenosine blockers (adenosine and dichloroadenosine).

The Ca^{2+} channel blockers, e.g., 1,4-dihydropyridine (nifendipine, IC_{50} 9×10^{-6} M), negative allosteric regulators of 1,4-dihydropyridine binding (verapamil, IC_{50} 10^{-6} M, and D-600, IC_{50} 5×10^{-6} M), and prenylamine (IC_{50} 8×10^{-6} M) partially displace the ^{125}I-DMABI to its binding site (30–50% of maximal inhibition). By contrast, the positive allosteric regulators of 1,4-dihydropyridine binding, diltiazem and diphenylalkylamine, e.g., cinnarizine and bepredil, do not compete with ^{125}I-DMABI for binding to crude intestinal membranes.

The isoquinoline derivatives that compete with ^{125}I-DMABI for binding to intestinal membranes were also tested for their ability to inhibit the contraction of intestinal smooth muscle. It appears that all derivatives recognized by the ^{125}I-DMABI-binding site inhibit muscle contraction. Moreover, derivatives which have no effect on smooth muscle contraction do not compete with ^{125}I-DMABI binding. When the K_i values of derivatives for inhibiting ^{125}I-DMABI were plotted against their IC_{50} values in inhibiting smooth muscle contraction (Fig. 6), a



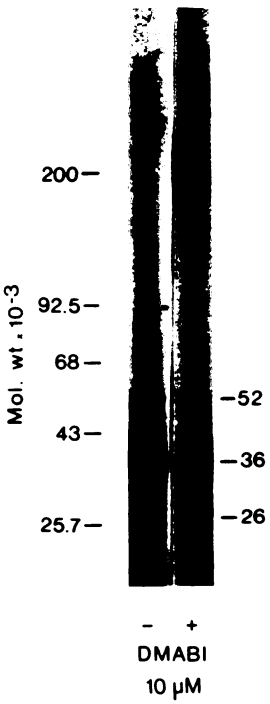


Fig. 8. Photoaffinity labeling of ^{125}I-DMABI and dose response of unlabeled DMABI in inhibiting the covalent labeling by ^{125}I-DMABI into rat intestinal membranes. Rat intestinal membranes were incubated with ^{125}I-DMABI (5–10 nM) in the absence and presence of unlabeled DMABI (10^{-8}–10^{-4} M), washed, photolyzed, and electrophoresed on 5–20% polyacrylamide slab gels as described under Materials and Methods. The results shown are identical with those of two other experiments. At *right* are the molecular weights (*Mol. wt*) of known proteins used as standards: α-chymotrysinogen (25.7 kD), ovalbumin (43 kD), bovine serum albumin (68 kD), phosphorylase B (92.2 kD), and myosin (220 kD). Autoradiographs of gel were scanned at 600 nm. The relative mobility of proteins in the gel is expressed relative to the mobility of the dye front. Ratio of the *M*, = 52,000:36,000:26,000 and that of specifically labeled peptides is 14:75:11.

highly significant correlation was observed, indicating that the structural requirements for both processes are very similar.

Covalent photoincorporation of ^{125}I-DMABI to its intestinal binding site. After 1 hr of incubation of ^{125}I-DMABI and intestinal crude membranes, the reversibility of ^{125}I-DMABI photoincorporation was tested by adding 100 μM unlabeled DMABI. After 1 hr of incubation in the dissociation medium, 75% of bound ^{125}I-DMABI remained bound to UV-treated membranes compared to 20% for untreated membranes (Fig. 7). This result indicated that ^{125}I-DMABI is irreversibly incorporated by photoaffinity cross-linking into intestinal membrane.

Crude intestinal membranes were incubated with ^{125}I-DMABI in the presence or absence of 0.01–100 μM unlabeled DMABI then submitted to UV irradiation, and finally analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiograms show that ^{125}I-DMABI is covalently bound to several membrane components (Fig. 8). Three protein bands were photolabeled, corresponding to ^{125}I-DMABI-protein complexes of 52,000 (14%), 36,000 (75%), and 26,000 (11%) molecular weight. These bands were protected from radioactive labeling by the presence of unlabeled DMABI in the initial incubation. This result suggests that they are involved in the specific binding of ^{125}I-DMABI to crude intestinal membranes.

Microdensitometric scanning of autoradiographs (Fig. 8) and integration of the peaks obtained (not shown) permit further analysis of the characteristics of the 52,000, 36,000, and 26,000 molecular weight components. Labeling of the three protein bands is reduced by unlabeled DMABI in the same concentration range, between 10^{-8} and 10^{-4} M; half-maximal reduction was obtained at 0.2–0.3 μM for each band.

Discussion

Our results document the usefulness of ^{125}I-DMABI for identifying and characterizing, by photoaffinity labeling, mammalian isoquinoline-binding peptides. As noted, ^{125}I-DMABI has all of the desirable features of photoaffinity ligand, including high specific radioactivity and high pharmacological selectivity for the binding site. Thus, this new probe should greatly facilitate work on the structure of the isoquinoline-binding site. The only ligand available to date has been [^{3}H]papaverine. This ligand lacks many desirable features necessary for successful identification and labeling of the isoquinoline-binding site in crude membrane preparation from various tissues. Indeed, [^{3}H]papaverine not only has a low specific activity (15) but cannot be used for photolabeling of the isoquinoline-binding site.

In the last few years several radioaffinity probes have been developed for characterizing hormone and drug receptors. The *p*-azido *m*-[^{125}I]iodobenzyl or phenyl derivatives have been successful for characterizing and labeling α_1-adrenoreceptors (22, 23), β-adrenoreceptors (24, 25), adenosine receptors (26, 27), and dopamine receptors (28). The chemical strategy consisted in the introduction of an arylamine moiety into identified ligand molecules, which not only preserved affinity and specificity for drug receptors, but also allowed the compounds to be radioiodinated. A similar approach has been taken to develop a highly specific photoaffinity probe for the isoquinoline-binding site. The presence of 4-benzyl in the isoquinolic series synthesized in our laboratories which can be replaced in *para* position is a good opportunity for directly synthesizing a radiophotoaffinity probe. The design of ^{125}I-DMABI is based on the structure of the highly relaxating molecule described in the 4-benzyl isoquinoline series, i.e., the PV-2 or 6,7-dimethoxy 4-(4′-chlorobenzyl) isoquinoline (16, 17, 29). It has been documented that the replacement of the 4′-chlorobenzyl by different radicals does not alter the pharmacological activity (17, 29). Indeed, the synthesis of DMABI yields a compound which retains relaxating activity and allows the radioiodination with high specific activity. Moreover, this compound offers the pos-

sibility for converting the arylamine to arylazide, giving rise to a potential photoaffinity probe of DMABI-binding sites. However, the present results indicate that the arylamine isoquinoline derivative itself can be directly incorporated in the membrane proteins upon UV irradiation. Direct photolabeling of membrane proteins has been similarly obtained with labeled compounds containing aromatic moiety such as benzodiazepine (30, 31), nitrendipine (32), chlorpromazine and trifluoperazine (33), trimethisoquine and phencycline (34), cytochalasin B (35), and other ligands, e.g., nucleotides (36), cholecystokinin (37), and glucagon (38). The mechanism by which those compounds are covalently linked to protein upon UV irradiation is poorly understood (39, 40); whatever the mechanism of photoincorporation, this method is simple and eliminates further important chemical modifications which might lose the pharmacological activity of isoquinolines.

Under nonphotolyzing conditions, ^{125}I-DMABI binds in a reversible manner to rat intestinal membranes in two populations of binding sites. Competitive binding studies using various isoquinoline derivatives and well defined drugs demonstrate that ^{125}I-DMABI has the specificity of an isoquinolic ligand. As judged by our results, two points are important for pharmacologically characterizing the isoquinoline-binding site. 1) ^{125}I-DMABI does not bind to the cAMP-PDE in intestinal membrane. Indeed, cAMP does not compete with ^{125}I-DMABI, cAMP-PDE inhibitors structurally different from isoquinoline do not displace ^{125}I-DMABI, and molecular weights of labeled membranous components (52,000, 36,000, and 26,000) are different from that of cAMP-PDE (52,000–59,000) (41–43). 2) The relation of the membranous isoquinoline-binding site with Ca^{2+} cellular fluxes, suggested by Kimura *et al.* (15) using [^{3}H] papaverine as probe, is confirmed by our results. ^{125}I-DMABI binding is totally inhibited by the Ca^{2+} chelating agent EGTA. Moreover, some Ca^{2+} antagonists partially displace ^{125}I-DMABI, and it is particularly interesting that verapamil and D-600, referenced as papaverine derivatives (44), are competing agents. However, it is clear that the ^{125}I-DMABI does not bind to the Ca^{2+} channel. Indeed, by contrast to what was observed for the Ca^{2+} channel (45–50), the ^{125}I-DMABI binding is relatively heat sensitive, not sensitive to alkylating and reducing agents and not regulated by GTP and ATP. Moreover, the peptides labeled by ^{125}I-DMABI are different from those observed with Ca^{2+} channel ligands (142,000, 45,000, and 33,000–32,000) (32, 51–54).

The highly significant correlation observed between competitive binding and the relaxant effect of various isoquinoline derivatives demonstrates that: 1) the isoquinoline-binding site is involved in the relaxation of intestine, and 2) a high structural requirement is necessary to recognize the isoquinoline-binding site. For instance, alteration of the C-6 and C-7 methoxy groups (hydroxy versus methoxy) drastically affects activities, as well as occupancy, of the C-1 and N^2 positions in the isoquinoline moiety. The benzyl group at the C-4 position plays an important role in the configuration related to the isoquinoline nucleus.

Following on investigations of the mode of action of analgesic morphine alkaloids that led to the discovery of endogenous morphine-like peptides in the brain (55, 56), the discovery of new binding sites for pharmacological agents in the organism raises the question of whether endogenous compounds are able to interact with the binding sites. At the present time, nonendogenous 6,7-dimethoxy isoquinoline-like substances have been identified, but the radioreceptor assay developed in the present study should be an invaluable tool for screening such substances.

In summary, the newly synthesized arylamine isoquinoline, ^{125}I-DMABI, is a promising tool for studying the molecular properties of the isoquinoline action site. It should greatly facilitate the elucidation of isoquinoline mechanisms of action in smooth muscle relaxation.

Acknowledgments

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