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CHARACTERIZATION OF SOLUBILIZED BRADYKININ B_2 RECEPTORS FROM SMOOTH MUSCLE AND MUCOSA OF GUINEA PIG ILEUM

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Abstract—Bradykinin (BK) B_2 receptors in guinea pig ileum were characterized in both membrane and soluble form. [^{3}H]BK bound to a single class of sites with almost identical affinities in membranes prepared from the longitudinal muscle, circular muscle and mucosal layers of the ileum. The pharmacology of the binding in the distinct layers was indistinguishable. The detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) maximally solubilized nearly 80% of membrane binding activity in a very stable conformation. In soluble preparations, [^{3}H]BK labeled a single class of sites but with about 10-fold lower affinity. The affinities of BK analogs in competition studies were similarly reduced. There was no difference in the pharmacology of the binding in soluble receptors prepared from the different layers of the ileum. The results show that the ileum is a good source of solubilized B_2 receptors and that the receptors in the smooth muscle and the mucosa are very similar.

Bradykinin (BK‡) displays numerous biological activities and may be an important mediator of pain and inflammatory responses [1]. The cell membrane receptors for BK and the related endogenous kinins, Lys-BK and Met,Lys-BK, are classified as being of either the B_1 or B_2 type [1]. BK-B_1 receptors are distinguished by their sensitivity to des-Arg^9-kinins, these peptides being virtually without activity at B_2 receptors. It appears that most of the physiological actions of BK involve B_2 receptor activation, although B_1 receptors may play a significant role in mediating kinin effects in traumatized tissue. Agonist stimulation of B_2 receptors has been shown to lead to increases in phosphoinositide hydrolysis and eicosanoid biosynthesis, both of which occur in most tissues that possess these receptors. It is not known whether different receptors are associated with these different transduction mechanisms but there is evidence that B_2 receptors can activate phospholipase A_2 as well as phospholipase C via GTP-binding proteins [2]. Given the diversity of cell types in which B_2 receptors are present, it would not be surprising if subclasses of these exist and there have been reports suggesting B_2 receptor heterogeneity [3, 4]. Recently, Farmer *et al.* [5, 6] provided definitive evidence of a novel BK receptor in guinea pig airways which they referred to as B_3 although it does fall into the above definition of a B_2 receptor. In view of this, future studies of BK receptors will certainly be focused on the characterization of their biochemical and molecular properties. The ileum of several species has been used to investigate the pharmacology and physiology of BK [1]. Receptors in this tissue are of the B_2 type and mediate BK-induced smooth muscle contraction as well as kinin stimulation of mucosal electrolyte secretion [7, 8]. In this report we describe the solubilization of BK receptors from guinea pig ileum. The results reported here show that guinea pig ileum is an excellent source material for stable solubilized B_2 receptor and that receptors in both the muscle and mucosal layers are very similar.

MATERIALS AND METHODS

Materials. Male Hartley guinea pigs (300–600 g) were obtained from Hazelton Research Products (Denver, PA). [^{3}H]Bradykinin (96–105 Ci/mmol) was purchased from DuPont NEN (Boston, MA). BPP5a was from Bachem Bioscience Inc. (Philadelphia, PA), while BK, its analogs and all other peptides were from Peninsula Laboratories (Belmont, CA). CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was obtained from Calbiochem (La Jolla, CA). Soybean trypsin inhibitor, digitonin, aprotinin, bacitracin, leupeptin, benzamidine, chymostatin, 1,10-phenanthroline, iodoacetamide and TES (*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid) were from Sigma (St. Louis, MO). Enalaprilat (MK-422) was from Merck Sharp & Dohme (Rahway, NJ). All other reagents used for these studies were of the highest grade commercially available.

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‡ Abbreviations: BK, bradykinin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; and EGTA, ethyleneglycol-bis-(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Tissue and membrane preparation. Animals were anesthetized with ether and killed by decapitation. The entire ileum was removed, dissected free of fat, and thoroughly flushed with cold isotonic saline. The longitudinal muscle was obtained by the method of Rang [9]. Approximately 10- to 15-cm lengths of tissue were stretched over a 1-mL pipet and a piece of gauze was used to separate the longitudinal muscle from the underlying tissue by gently rubbing away from the mesenteric border. The mucosa was obtained by everting the tissue and scraping with a glass slide until the tissue was completely translucent. The tissue remaining following the removal of the longitudinal muscle and mucosal layers was taken as the circular muscle. For membrane binding assays each tissue was homogenized, using a Polytron (Brinkmann Instrument Co., Westbury, NY), in 20 vol. of ice-cold 25 mM TES buffer (pH 6.8 at room temperature) containing 1 mM phenanthroline and centrifuged at 48,000 *g* for 20 min. The membrane pellet was washed one time by rehomogenization in 20 vol. of fresh buffer followed by centrifugation as before. The final pellet was resuspended in membrane binding assay buffer (buffer above with 0.1% protease free bovine serum albumin, 5 μM MK-422, and 100 μg/mL bacitracin) using a motor driven Potter–Elvehjem tissue grinder. Membranes used for solubilization studies were similarly prepared except that the homogenization buffer was supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.5 mM ethyleneglycol-bis-(β-aminoethyl)*N,N,N'N'*-tetraacetic acid (EGTA) and 1 mM benzamidine (buffer A). Following the second centrifugation, the membranes were resuspended to a concentration of approximately 10 mg protein/mL and stirred for 1 hr at room temperature with 1 mM iodoacetamide. The suspension was centrifuged for 20 min at 48,000 *g* and the membrane pellet was resuspended to 15 mg protein/mL with solubilization buffer (buffer A with 50 μg/mL bacitracin, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL chymostatin, 0.2 mg/mL sodium azide and 100 mg/mL glycerol). The membranes were stirred on ice and solid CHAPS was slowly added to yield a final concentration of 10 mM. After 1 hr the solution was centrifuged at 100,005 *g* for 75 min and the clear supernatant was recovered and either frozen at −80° or used for assay. Protein determinations were performed by the method of Bradford [10] using bovine IgG as standard.

Binding assays. Membrane binding assays followed the procedure of Manning *et al.* [11]. [^{3}H]BK was incubated for 75 min with 0.5 mg tissue wet weight (15 μg membrane protein) in a final volume of 1 mL. The assay was terminated by filtration over 0.1% polyethyleneimine (PEI)-soaked (2 hr) Whatman GF/B filters using a Brandel cell harvester. The tubes were rinsed twice with 4 mL of ice-cold 10 mM TES (pH 6.8) and filter bound radioactivity was quantitated by liquid scintillation spectrometry. Nonspecific binding was determined by incubating samples in the presence of 1 μM BK and represented less than 5% of the total binding at 50 nM [^{3}H]BK.

Binding assays with solubilized receptor were performed in 0.5 mL of buffer A with 2.5 mM CHAPS, 50 μg/mL bacitracin, 5 μM MK-422 and 0.06% crude soybean phospholipid. Typically, 5–7 μL (30–50 μg protein) of the soluble receptor preparation was added to each tube. Incubations were for 60 min at room temperature followed by filtration over PEI (0.3%) soaked filters as described for the membrane receptor. Nonspecific binding was about 5% of the total binding at 100 pM [^{3}H]BK. Competition and saturation experiments were analyzed using the EBDA program of McPherson [12] or GraphPad (ISI Software, Philadelphia, PA).

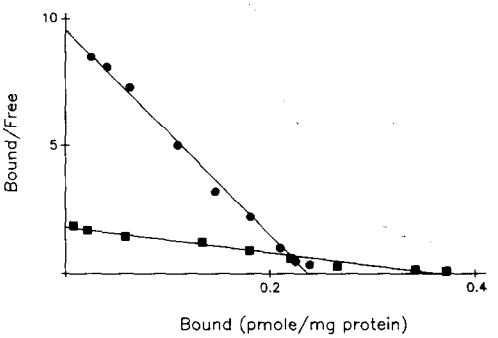

Fig. 1. Scatchard plot of [^{3}H]BK binding to membrane (●) and solubilized (■) receptors prepared from whole guinea pig ileum. The binding parameters generated for each preparation are listed in Table 1. The figure represents data from a single experiment which was reproduced two additional times with similar results.

RESULTS

Membrane [^{3}H]*BK binding.* [^{3}H]BK saturation binding experiments were performed with membranes prepared from whole ileum as well as the separated muscle and mucosal layers. Analysis of the untransformed data by nonlinear regression or using the method of Scatchard [13] showed that in each preparation [^{3}H]BK labeled a single class of sites with similar affinities (Fig. 1, Table 1). The similarity between receptors in muscle and mucosa was evident in competition studies as well. As shown in Table 2, the inhibitory potencies of both agonist and antagonist analogs of BK were nearly the same in each membrane preparation. Results using circular muscle are not shown but they were very similar to the values listed for the longitudinal muscle and mucosal preparations. The calculated Hill coefficients in these experiments were near one for each of the competitors.

Solubilized receptor studies. Using the procedure described in Materials and Methods, 50–60% of the [^{3}H]BK binding activity of whole ileum could be extracted from membranes with a slight increase in specific activity (Fig. 1). An additional 20% of the original activity could be recovered as soluble receptor if the initial detergent-treated pellet was reextracted with 10 mM CHAPS although this was not typically done. Similar solubilized receptor yields were obtained when the muscle mucosal layers were individually solubilized. The solubilized [^{3}H]BK

Table 1. [^{3}H]BK binding parameters in membrane and solubilized receptor preparations

Tissue	Membranes		Solubilized	
	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)
Whole ileum	18 ± 3	220 ± 10	186 ± 15	321 ± 19
Longitudinal muscle	21 ± 3	182 ± 13	172 ± 12	341 ± 11
Circular muscle	20 ± 4	209 ± 12	191 ± 17	391 ± 24
Mucosa	18 ± 2	241 ± 14	196 ± 13	318 ± 15

Saturation experiments were performed using [^{3}H]BK concentrations from 4 pM to 400 pM for the membranes and 10 pM to 1 nM for the soluble receptor. The results are means ± SEM of at least three separate determinations.

Table 2. Inhibition of [^{3}H]BK binding to longitudinal muscle and mucosal membranes

Inhibitor	K_i (nM)			
	Membranes		Solubilized	
	Muscle	Mucosa	Muscle	Mucosa
Agonists				
BK	0.023	0.018	0.20	0.19
Lys-BK	0.032	0.027	0.67	0.76
Met,Lys-BK	0.071	0.059	1.2	1.3
Tyr^8-BK	0.15	0.15	2.2	4.0
Tyr^5-BK	3.1	2.4	26	29
Des-Arg^9-BK	14,600	19,200	$>10^5$	$>10^5$
Antagonists				
D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK	2.1	2.9	31	34
D-Arg[Hyp^{2,3},Thi^{5,8},D-Phe^7]BK	3.3	3.1	38	49
Lys,Lys[Hyp^3,Thi^{5,8},D-Phe^7]BK	6.5	7.7	108	106
[Thi^{5,8},D-Phe^7]BK	25	19	214	185
Des-Arg^9[Leu^8]BK	21,600	18,300	$>10^5$	$>10^5$

Competition experiments were performed with at least eight concentrations of inhibitor. The K_i values were generated using the K_d values for [^{3}H]BK in Table 1 by the equation of Cheng and Prusoff [14]. The results are from at least three experiments for each inhibitor in which the SEM was not greater than 10% of the above values. Thg following peptides did not affect either membrane or soluble [^{3}H]BK binding at 10 μM: oxytocin, angiotensin I or II, neurotensin, substance P, endothelin-1, cholecystokinin and BPP5a.

binding activity was not sedimented by high speed centrifugation and passed through a 0.2 μm membrane filter. The soluble receptor was most easily and efficiently assayed using PEI-treated glass fiber filters [15] and yielded quantitatively the same results as gel-filtration methods (Sephadex G-25, data not shown). The optimal concentration of CHAPS in the binding assay was 2 to 2.5 mM and at this detergent concentration the inclusion of 0.006% soybean phospholipids increased specific binding by 70%. There was no inhibition of binding by any of the protease inhibitors used in the assay. Less than 10% of the soluble binding activity was lost after 48 hr at 4° and the extract appeared to be almost indefinitely stable (>10 months) when stored at −80° in the solubilization buffer. Of several other detergents tested only digitonin (1%) successfully solubilized [^{3}H]BK binding activity but the recovery was less than with CHAPS.

The identity between membrane BK receptors and the solubilized binding activity was suggested in several ways. Since [^{3}H]BK binding to membrane receptors exhibits a low pH optimum [16], this property was examined. Figure 2 shows that the pH dependencies for membrane and soluble ileum binding were nearly identical. It was also found that monovalent cations had the same rank order of potencies for inhibition of [^{3}H]BK to membrane and soluble sites although they were somewhat less active in the membrane assay. The IC_{50} values (mM, mean ± SEM, N = 3) for Li^+, Na^+ and K^+ (chloride salts) were, respectively, 31 ± 2, 37 ± 3 and 48 ± 3 at the soluble receptor and 59 ± 3, 65 ± 4 and 95 ± 7 in membranes. Saturation binding experiments

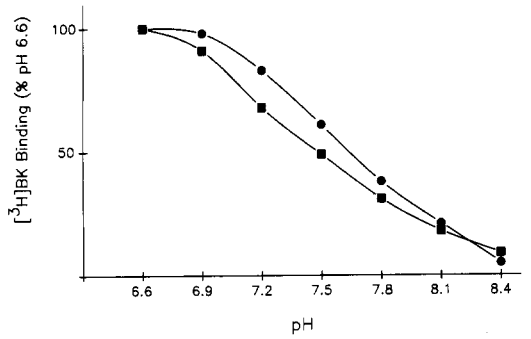

Fig. 2. The pH dependency of [^{3}H]BK binding to soluble (●) and membrane (■) receptors from whole guinea pig ileum. The data are from a single experiment which was reproduced two additional times with similar results. The points represent the means of triplicate determinations that varied by less than 10%.

showed that [^{3}H]BK bound to the solubilized receptor from whole ileum with reduced affinity (Fig. 1, Table 1). As in membranes though, analysis of the curves indicated that the radioligand labeled a single class of noninteracting sites. Similar results were obtained when the muscle and mucosal layers were solubilized separately (Table 1). The shift in affinity was equally apparent in competition studies and was not restricted to BK since all of the analogs used to pharmacologically characterize the binding exhibited reduced potencies (Table 2). The Hill coefficients in these experiments were essentially one. The similarity between smooth muscle and mucosal BK receptors was again evident in the nearly identical potencies exhibited by the peptides in inhibiting binding to solubilized sites in each preparation.

DISCUSSION

Solubilization and characterization of kinin B_2 receptors have been reported using bovine uterine myometrium [17], rat uterus [18], NG108-15 cells [18] and human fibroblasts [19]. In these previous studies, the yields of soluble receptors were low and the binding activity of the preparations was observed to deteriorate relatively quickly. We have shown that guinea pig ileum B_2 receptors can be solubilized in good yield and the [^{3}H]BK binding activity is very stable. The ileum was chosen for this investigation because it has been used extensively to pharmacologically characterize kinin receptors and a single animal can provide a relatively large amount of tissue. Thus, a good solubilization procedure would allow the preparation of sufficient receptor for biochemical characterization. Yet, the ileum is heterogenous and could possess a mixed receptor population, presenting problems in subsequent studies. However, an earlier report had detected no significant differences in [^{3}H]BK binding to smooth muscle and mucosal membranes [7] and the results presented here further demonstrate that B_2 receptors in the separate layers are very similar, if not identical. [^{3}H]BK was shown to bind with the same high affinity to membranes prepared from each layer and there were no differences in the pharmacology of the binding between the regions. While differences may not have been expected between the receptors localized in the longitudinal and circular smooth muscle, it is interesting that these receptors appear to be identical to the mucosal B_2 receptors as they subserve different physiological functions (contraction vs electrolyte secretion). It is also significant that there was no suggestion of receptor heterogeneity within the mucosa as BK receptors are distributed on both epithelial cells and nonepithelial cells in this portion of the ileum [7].

The most striking result was the large reduction in [^{3}H]BK binding affinity upon solubilization with CHAPS. While this could suggest that the soluble sites are unrelated to those labeled in membranes, this does not appear to be the case. First, the yield of soluble [^{3}H]BK binding is consistent with the density of membrane sites as about 80% of the latter could be obtained in soluble preparations. Second, it was also found that the pH dependency of [^{3}H]-BK binding was the same in the CHAPS-extracted material as in membranes. The affinities of documented B_2 receptor agonists and antagonists were also reduced although to somewhat varying degrees. Lastly, the most likely candidate for a spurious binding site in the solubilized preparations would be a peptidase or proteinase. This does not seem possible in that no peptides unrelated to BK, including those acting as proteinase inhibitors, were found to interfere with soluble [^{3}H]BK binding.

Recently, expression cloning has identified a rat uterine bradykinin B_2 receptor comprised of 366 amino acids [20]. While molecular biological approaches to receptor identification and characterization are inclined to obviate the need for the more traditional biochemical methods, the latter studies may yet provide valuable information. For instance, although having an amino acid sequence may help define sites for post-translation processing of receptor, only extraction of the protein from its tissue of origin can identify which, and to what extent, such modifications actually take place. For this, extraction and analysis of the native receptor are essential. Thus, the ability to solubilize stable receptor in good yield as a prelude to isolation is still of importance.

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