Localization of Specific Binding Sites for Atrial Natriuretic Factor in Peripheral Tissues of the Guinea Pig, Rat, and Human

CHRISTOPHER R. MANTYH, LAWRENCE KRUGER, NICHOLAS C. BRECHA, AND PATRICK W. MANTYH

SUMMARY Specific, high affinity atrial natriuretic factor (ANF) binding sites were identified and localized by autoradiographic techniques in peripheral tissues of the guinea pig, rat, and human. In the guinea pig kidney, high concentrations of ANF binding sites were located in the glomerular apparatus, outer medulla, and small renal arteries. Other peripheral tissues containing ANF binding sites included the zona glomerulosa of the adrenal cortex, the smooth muscle layer of the aorta and gallbladder, the lung parenchyma, the posterior lobe of the pituitary, the ciliary body of the eye, and the leptomeninges and choroid plexus of the brain. The distribution of ANF binding sites in the rat and human kidney was nearly identical to those seen in the guinea pig kidney; high concentrations were present in the glomerular apparatus, outer medulla, and small renal arteries. These results are consistent with earlier physiological and pharmacological studies that suggested that ANF plays a functional role in the regulation of extracellular fluid volume and blood pressure. There appears to be little species variation in the location and concentration of renal ANF binding sites, suggesting that, at least in the kidney, the results in experimental animals are relevant to the actions of ANF in humans. The finding that ANF binding sites were stable and present in high concentrations in human postmortem kidneys further suggests that these tissues may be amenable to testing for the involvement of ANF receptor dysfunction in diseases such as hypertension and congestive heart failure.

KEY WORDS ciliary body • atriopeptins • receptors • kidney • adrenal • smooth muscle • choroid plexus • hypertension

A wide range of physiological experiments have indicated the existence of humoral factors that stimulate natriuresis and diuresis. Extracts of heart atria, but not ventricular tissue, have been shown to exhibit natriuretic and diuretic activity, and studies of these crude extracts have indicated that the kidney is a major target tissue. The effect of atrial natriuretic factor (ANF) on renal salt excretion is of particular interest since extracellular fluid volume is regulated by the balance between sodium intake and excretion, and since a defect in renal sodium excretion has been postulated to be an underlying abnormality in some patients with hypertension. Recently, a family of peptides known as ANFs has been isolated, sequenced, and cloned. These peptides appear to have a common precursor and are released in response to changes in intra-atrial pressure and stretch of the atrial wall. Previous homogenate binding assays have demonstrated specific, high affinity receptors in rabbit and rat kidney and aorta and bovine adrenal glomerulosa cells. These observations suggest that ANFs released from the atria play a role in the regulation of fluid volume, electrolyte balance, and blood pressure.

In the present study, we examined several peripheral tissues in the guinea pig, rat, and human to determine if they contain specific ANF binding sites that may correspond to functional ANF receptors. The objectives were threefold: 1) to assess which peripheral
organs express specific ANF binding sites and thus are possible target tissues for circulating ANF; 2) to define whether the distribution and concentration of ANF binding sites in experimental animals are similar to those in humans; and 3) to determine the stability of the ANF binding sites in human postmortem material.

Materials and Methods

The quantitative autoradiographic receptor binding technique was used to identify the specific ANF binding sites. The incubation conditions used are a modification of a previous homogenate receptor binding study. Male guinea pigs (weight, 500 g; Simonsen Labs, Gilroy, CA, USA) and Sprague-Dawley rats (weight, 200 g; Simonsen Labs) were decapitated, and the peripheral tissues were removed, embedded in cryostat, serially sectioned at 30 μm on a cryostat at −20°C, thaw-mounted on gelatin-coated microscope slides, and stored at −20°C until use. For analysis of the human material, postmortem kidneys were obtained from a 57-year-old man who died of myocardial infarct, a 61-year-old man who died of cerebral aneurysm, and a 27-year-old man who died of head injury. The human tissue was obtained 12, 60, and 65 hours after death, respectively, and processed as described.

In initial experiments, we determined the optimal incubation conditions for ANF receptor binding in the guinea pig kidney in order to maximize the specific/nonspecific binding ratios. To measure the specific/nonspecific binding ratios, guinea pig kidney sections were scraped from the slide with a razor blade and placed in a counting tube and the radioactivity was determined using a gamma counter. Nonspecific ANF binding was defined as that 123I-labeled ANF that was not displaced by an excess (1 μM) of cold ANF. After extensive trial and error, it was determined that the following incubation conditions consistently resulted in the highest specific/nonspecific binding ratios while retaining optimal optical resolution of the binding sites.

The slide-mounted tissue sections were first placed in a preincubation bath at 25°C containing 0.005% (vol/vol) polyethyleneimine (Sigma Chemical, St. Louis, MO, USA) and 50 mM N-2-hydroxethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer for 10 minutes. They were then incubated in 50 mM HEPES, 5 mM MgCl₂, 0.3% bovine serum albumin, 0.1% bacitracin, and 150 pM 123I-ANF at 25°C for 45 minutes. For rat and guinea pig tissue, 125I-rat ANF (Amer sham, Chicago, IL, USA) was the radioligand, and for the human tissue we used 125I-human ANF (Amer sham). The slide-mounted tissue sections were then washed three times (5 minutes each) at 4°C in 50 mM HEPES, 5 mM MgCl₂, 0.3% bovine serum albumin, and 0.1% bacitracin. Finally, the slides were rinsed twice for 5 seconds at 4°C in distilled water. Control slides were incubated similarly except for the second step, where either 1 μM of cold rat ANF (1–28) (Bachem, Torrance, CA, USA), cold human ANF (1–28) (Bachem), or an unrelated peptide (substance P, somatostatin 1–14, sulfated cholecystokinin-8, or thyrotropin releasing hormone; all from Bachem) was added. The slides were placed in apposition to LKB Ultrascan (Bromma, Sweden) for 1 week and then developed in Kodak D-19.

The autoradiograms in the figures are enlargements of the LKB Ultrascan negatives. To identify the cellular location of the binding sites, the sections were stained with hematoxylin and eosin and coverslipped with Permount. After development and fixation as just described, the optical densities of the autoradiograms were determined by projecting the autoradiograms at 20 × on a white, horizontal surface and quantifying the density of the projected image with a photocell connected to a digital voltmeter, as described by Rainbow et al. This densitometer consisted of a Sharp BS-500A silicon blue photodiode (Paramus, NJ, USA) connected to a Radio Shack voltage amplifier (Los Angeles, CA, USA). At 20 ×, the resolution of the device corresponded to a region 50 μm in diameter on the projected sections. Previous experiments had established that the LKB film does not respond linearly to a linear increase in radioactivity. We therefore constructed a series of standards, exposed these to the LKB film, developed and fixed the film, measured this film densitometrically, and used these values with the Texas Instruments (Lubbock, TX, USA) automatic curve-fitting program (Hewlett-Packard, Palo Alto, CA, USA) to obtain an equation that described the film’s characteristics. Mean raw density values for the concentration of ANF binding sites were then obtained from a minimum of five separate readings and placed in this equation to correct for the nonlinearity of the LKB film. Thus, all values listed in Table 1 are corrected optical densities such that a doubling of density values in this table corresponds to a doubling in the concentration of ANF binding sites in the peripheral tissues.

Results

Specific, high affinity ANF binding sites were demonstrated in rat, guinea pig, and human peripheral tissues using the autoradiographic technique and 125I-rat ANF (1–28) for rat and guinea pig tissue, and 125I-human ANF (1–28) for the human tissue (Table 1). Initial scrape-off experiments in the guinea pig kidney revealed that greater than 75% of the total binding was specific. No inhibition of specific binding was observed in adjacent sections where 1 μM of unrelated peptide including substance P, somatostatin 1–14, cholecystokinin-8, or thyrotropin releasing hormone was added to the incubation medium. These observations are consistent with previous membrane homogenate studies and indicate that the two techniques probably are identifying the same specific, high affinity ANF binding sites that appear to correspond to the ANF receptor.

In the guinea pig urogenital system, a high concentration of specific ANF binding sites was present in the glomerular apparatus (GA) of the kidney (Figure 1).
Comparison of the location and density of ANF binding sites indicates that of the several thousand GA specimens we have examined, all have a similar high concentration of ANF binding sites. The smooth muscle layer of the small renal arteries also had a high concentration of ANF binding sites (see Figure 1). The other region of the kidney with a high concentration of ANF binding sites was the outer medulla, which corresponds to the location of the cortical collecting tubules (see Figure 1). Other areas of the urogenital system examined displayed a negligible level of ANF binding sites, including all layers of the bladder, testes, vas deferens, seminal vesicles, uterus, and fallopian tubes.

In rat (Figure 2) and human (Figure 3) kidney, the distribution of specific ANF binding sites was almost identical to that seen in guinea pig kidney. High concentrations of ANF binding sites were present in the GA, outer medulla, and renal arteries. As with the guinea pig, of the several thousand GA specimens examined in either the rat or human, all displayed a high concentration of ANF binding sites.

In the guinea pig endocrine system, both the adrenal gland and the posterior pituitary revealed a substantial concentration of ANF binding sites. In the adrenal, ANF binding sites were confined to the zona glomerulosa of the adrenal cortex (see Figure 1). All other layers of the cortex and medulla showed a negligible concentration of ANF binding sites. In the pituitary, only the ventral aspect of the pars nervosa (posterior pituitary) contained a significant concentration of binding sites in contrast to the negligible concentration in the dorsal aspect of the pars nervosa, pars distalis, and pars intermedia (Figure 4). The rat adrenals displayed a distribution of binding sites confined to the zona glomerulosa (see Figure 2) that was nearly identical to that found in the guinea pig adrenals (see Figure 1).

In the guinea pig cardiovascular system, a moderate concentration of binding sites was seen predominantly in the smooth muscle layer of the aorta (Figure 5). The ANF binding sites were also present in the lung parenchyma (Figure 6), with no apparent proximal-distal concentration gradient. Negligible concentrations of binding sites were present in all other layers of the aorta, all areas of the right and left atrium and ventricles, pulmonary arteries and veins, trachea, bronchi, bronchioles, and larynx.

In the guinea pig gastrointestinal system, only the longitudinal muscle layer of the distal colon had a measurable level of ANF binding sites. All other areas of the gastrointestinal tract examined, including all layers of the esophagus, stomach, duodenum, ileum, jejunum, cecum, proximal colon, and rectum, had negligible concentrations of binding sites. The only other component associated with the digestive apparatus that contained a high concentration of ANF binding sites was the smooth muscle layer of the gallbladder (Figure 7). Negligible levels of binding sites were present in all other layers of the gallbladder, spleen, liver, pancreas, tongue, and salivary glands.

The ciliary body in the eye of the guinea pig displayed the highest concentration of ANF binding sites of all the tissues examined in guinea pig, rat, or human (Figure 8). These binding sites were clearly concentrated in the epithelium of the ciliary body; the other aspects of the ciliary body displayed negligible levels.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Optical Density</th>
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<tr>
<td>Urogenital system</td>
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<tr>
<td>Kidney</td>
<td></td>
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<tr>
<td>Glomerular apparatus</td>
<td>81.9 ± 5.7</td>
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<tr>
<td>Renal arteries (smooth muscle)</td>
<td>52.0 ± 4.0</td>
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<tr>
<td>Outer medulla</td>
<td>53.1 ± 2.3</td>
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<tr>
<td>Endocrine system</td>
<td></td>
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<tr>
<td>Pituitary gland (posterior lobe)</td>
<td>66.7 ± 3.1</td>
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<tr>
<td>Adrenal gland (zona glomerulosa)</td>
<td>54.2 ± 1.8</td>
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<tr>
<td>Respiratory system</td>
<td></td>
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<tr>
<td>Lung (parenchyma)</td>
<td>51.7 ± 1.3</td>
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<tr>
<td>Cardiovascular system</td>
<td></td>
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<tr>
<td>Aorta (smooth muscle)</td>
<td>42.1 ± 0.5</td>
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<tr>
<td>Eye</td>
<td></td>
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<tr>
<td>Ciliary body (epithelial layer)</td>
<td>100.0 ± 1.6</td>
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<tr>
<td>Choriocapillaris</td>
<td>45.8 ± 1.1</td>
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<tr>
<td>Gastrointestinal system</td>
<td></td>
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<tr>
<td>Colon (smooth muscle)</td>
<td>13.2 ± 1.7</td>
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<tr>
<td>Other systems</td>
<td></td>
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<tr>
<td>Gallbladder (smooth muscle)</td>
<td>43.1 ± 2.5</td>
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<tr>
<td>Choroid plexus</td>
<td>30.8 ± 0.7</td>
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<tr>
<td>Leptomeninges</td>
<td>38.2 ± 1.2</td>
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*The concentration is expressed as the corrected optical density of LKB Ultrasoft exposed to guinea pig, rat, and human tissue sections after incubation with either 125I-rat ANF (guinea pig and rat) or 125I-human ANF. Background labeling on the film has an optical density of 0.0, while the epithelial layer ciliary body of the guinea pig eye is defined as the highest optical value of 100.0.
†The values for the human are those of a 27-year-old man with a postmortem autopsy time of 65 hours.

Values are corrected optical densities ± SEM of at least five determinations throughout the area under examination. A dash indicates no data; negligible levels were found for all regions of the gastrointestinal tract examined, including all layers of the esophagus, stomach, duodenum, ileum, jejunum, cecum, proximal colon, and rectum, had negligible concentrations of binding sites. The only other component associated with the digestive apparatus that contained a high concentration of ANF binding sites was the smooth muscle layer of the gallbladder (Figure 7). Negligible levels of binding sites were present in all other layers of the gallbladder, spleen, liver, pancreas, tongue, and salivary glands.

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The choriocapillaris also contained a moderate concentration of binding sites. Of the intrinsic muscles of the eye, only the longitudinal muscle contained a significant concentration of binding sites (see Figure 8). All other layers of the eye, including the retina, sclera, lens, iris, and cornea, possessed a negligible concentration of ANF binding sites.

Other tissues in the guinea pig containing a distinctive concentration of specific ANF binding sites included the choroid plexus epithelium and the leptomeninges (i.e., pia and arachnoid) of the brain, as reported in a previous study. The concentration of ANF binding sites appeared to vary within the different regions of the choroid plexus; consistently high levels were present in the fourth ventricle, whereas markedly diminished or patchy concentrations were present in the lateral cerebral ventricles (Figure 9). Additional tissues examined that contained negligible concentrations of binding sites included all layers of the thyroid and the skin of the nose, hindpaw, and forepaw.

Examination of the concentration of ANF binding sites in the three human postmortem kidneys revealed no significant differences in either concentration or location based on the subject's age (27, 57, and 61 years) or time of autopsy (12, 60, and 65 hours after death).

**Discussion**

The distribution of ANF binding sites in the peripheral tissues examined in the present study appears consistent with the functional role generally ascribed to ANF in regulating fluid volume, electrolyte balance, and blood pressure. Previous studies have suggested that ANF acts directly on the kidney, because injection of ANF produces a marked increase in urine volume and an accompanying increase in sodium, potassium, and chloride excretion. This effect is consistent with the reports that specific ANF binding sites are found in tissue homogenates of rabbit and rat kidney and aorta, as well as in bovine adrenal glomerulosa cells.

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**Figure 1.** Dark-field photomicrographs of LKB film autoradiograms of a guinea pig kidney and adrenal section (a), incubated in the presence of 150 pM $^{125}$I-atrial natriuretic factor (ANF), and its serial pair (b), incubated in 150 pM $^{125}$I-ANF plus 1 μM ANF. In these and the following dark-field photomicrographs, $^{125}$I-ANF binding appears as white silver grains. Since nonspecific binding is defined as that $^{125}$I-ANF binding that remains in the presence of 1 μM ANF, Panel b is an image of the nonspecific binding. Thus, the specific binding in Panel a is obtained by subtracting the nonspecific binding in Panel b. A high concentration of receptors is present in the glomerular apparatus (GA), renal arteries (RA), and outer medulla (OM) of the kidney. The zona glomerulosa (ZG) is clearly outlined in the adrenal cortex. (Line bar = 1.5 mm.)
ANF + 1μM ANF

FIGURE 2. Dark-field photomicrograph showing the localization of atrial natriuretic factor (ANF) binding sites in a coronal section of the rat kidney and adrenal. Note the high concentration of ANF binding sites in the glomerular apparatus (GA), renal arteries (RA), outer medulla (OM), and zona glomerulosa (ZG). See Figure 1 for explanation. (Line bar = 1.5 mm.)

In the present study, guinea pig, rat, and human kidney revealed a very high concentration of specific binding sites localized to the GA, renal arteries, and outer medulla. The high concentration of ANF binding sites in the GA and outer medulla (corresponding to the location of the cortical collecting tubules) suggests that these regions are primary sites of ANF's natriuretic and diuretic actions on the kidney. The localization of high concentrations of specific ANF binding sites on renal arteries and moderate levels in the muscularis layer of the aorta suggests a direct action on vascular smooth muscle, accounting for ANF's effects on vascular resistance.25,29 Recent studies suggest that ANF acts directly on the adrenals since ANFs inhibit basal aldosterone release as well as block the release of aldosterone from isolated adrenal glomerulosa cells stimulated by adrenocorticotropic hormone and angiotensin II.31-33 The present report directly supports this view by demonstrating high concentrations of specific ANF binding sites in the zona glomerulosa of the adrenal cortex, the region where aldosterone is principally synthesized and secreted. Moderate concentrations of ANF binding sites in the posterior pituitary may account for the reported ANF stimulation of vasopressin release from the isolated posterior pituitary gland,36 but the significance of its presence in only a sector of this endocrine organ remains obscure and deserves further study.

The kidney, the ciliary body of the eye, and the choroid plexus of the brain are all involved in plasma filtration and in specialized active transport. The ciliary body is involved in the production of the vitreous fluid, and the choroid plexus is involved in the secretion of cerebrospinal fluid. The present finding of a distinctly localized, high concentration of ANF binding sites in these structures suggests that ANF also may be involved in regulating the environment and pressure of interocular and intracranial fluids.

In other peripheral tissues, however, it is not clear whether the ANF binding sites are generally involved in the regulation of fluid and electrolyte balance or whether they are involved in specific active transport functions. Thus, binding sites in the colon and gallbladder might be implicated in regulating the degree of water reabsorption by influencing the transit time of the meal and bile, respectively. In the lung, the high concentration of ANF binding sites might be related most logically to regulation of water reabsorption. Interestingly, the BIO 14.6 hamster, which is known to experience congestive heart failure, has a marked deficiency in the atrial content of its ANF.37 Coupled with the present finding, this observation may indicate that
FIGURE 3. Dark-field photomicrograph showing the autoradiographic localization of atrial natriuretic factor (ANF) binding sites in a coronal section of human kidney obtained from a 27-year-old man (autopsy time, 65 hours after death). Note the high concentration of binding sites in the glomerular apparatus (GA), renal arteries (RA), and outer medulla (OM). See Figure 1 for explanation. (Line bar = 1.7 mm.)

FIGURE 4. Light-field photomicrograph of a guinea pig pituitary stained with hematoxylin and eosin (H & E; a), and dark-field photomicrographs of LKB autoradiograms of the same section (b) and its serial pair (c). See Figure 1 for explanation. A moderate concentration of 125I-atrial natriuretic factor (ANF) binding sites can be seen in the pars nervosa (PN), while a negligible concentration of binding sites can be seen in the pars distalis (PD) and pars intermedia (PI). (Line bar = 0.6 mm.)
FIGURE 5. Light-field photomicrograph of a guinea pig aorta stained with hematoxylin and eosin (H & E; a), and dark-field photomicrographs of LKB film autoradiograms of the same section (b) and its serial pair (c). See Figure 1 for explanation. A moderate concentration of atrial natriuretic factor (ANF) binding sites can be seen in the tunica media (TM; smooth muscle layer), while a negligible concentration can be seen in the tunica intermedia (TI). (Line bar = 0.7 mm.)

FIGURE 6. Light-field photomicrograph of a guinea pig lung stained with hematoxylin and eosin (H & E; a), and dark-field photomicrographs of LKB film autoradiograms of the same section (b) and its serial pair (c). See Figure 1 for explanation. Note the heavy concentration of binding sites in the parenchyma (P), while no specific binding can be seen in the bronchus (Br) or the blood vessels (BV). (Line bar = 1.5 mm.)

The dysfunction in the BIO 14.6 hamster is due not only to the lack of ANF stimulation of natriuretic and diuretic response in the kidney but also to the lack of ANF-induced water reabsorption by the alveoli.

The demonstration of comparable ANF binding sites in human, guinea pig, and rat kidney suggests...
that, at least in the kidney, the physiological actions of ANF in experimental animals are similar to those seen in humans. The unexpected stability of ANF binding sites in the postmortem human kidney also suggests that human kidneys could be assessed for ANF receptor dysfunction in disease states such as hypertension and congestive heart failure. The high concentration and postmortem stability of ANF binding sites found in the human kidney indicate that this tissue should prove an excellent source for the extraction.
Figure 9. Dark-field photomicrograph of a horizontal section of the rat brain showing specific atrial natriuretic factor (ANF) binding sites in the olfactory bulb (OB), fasciculus retroflexus (fr), pia-arachnoid (p/a), and choroid plexus (cp). Specific binding sites were not observed in the cerebellum (Cb), caudate-putamen (CPu), or the hippocampus (H). (Line bar = 1.6 mm.)

purification, and sequence analysis of the human ANF receptor. While the present study provides information concerning the location and possible target tissues for ANF, it cannot indicate whether these tissues are occupied by circulating ANF or by locally synthesized ANF. Immunohistochemical data have revealed that in addition to the atrium, the salivary glands and several areas of the brain display cells containing ANF-like immunoreactivity. However, recent RNA blot hybridization experiments have suggested that the atrium is the only tissue examined that displays high levels (>0.5%) of the total messenger RNA (mRNA) coding for ANF. These histochemical and biochemical experiments are not necessarily in disagreement, since the RNA blot technique only detects very high levels of ANF mRNA. Therefore, tissues that synthesize relatively small amounts of ANF mRNA could very well be below the limits of detection with this technique but detectable with immunohistochemical techniques.

It is not clear whether all the binding sites identified in the present study correspond to functional ANF receptors. Previous experiments strongly suggest that several of these binding sites have the proper physiological location (i.e., kidney, adrenals, and aorta) and pharmacological characteristics to be considered ANF receptors. Before many of the tissues we have shown to express ANF binding sites (i.e., lung, gallbladder, ciliary body, choroid plexus) can be properly considered to express a functional ANF receptor, however, activation of these binding sites must be shown to produce a relevant physiological response.

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