Acta Med. Nagasaki 42 : 10−15

Assays of ^{125}I-Bolton-Hunter Substance P Binding Sites, Functional Sites of Tachykinin NK_1 Receptor : Quantitative Receptor Autoradiographic Method

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^{125}I-Bolton-Hunter labeled tachykinins, substance P, neurokinin A, and eledoisin have been used as radioligands to differentiate multiple tachykinin receptors[11,12,30]. ^{125}I-Bolton-Hunter substance P seems to be a radioligand predominantly recognizing NK_1 subtype of tachykinin receptors. As described here, using the quantitative receptor autoradiographic method, the binding sites specifically labeled with the radioligand can be vigorously analyzed. In addition to substance P, neurokinin A and neurokinin B known to be endogenously, new tachykinins, neuropeptide K[31] and neuropeptide R[32] have been isolated from the porcine brain and rabbit small intestine, respectively. Further characteristics of tachykinin receptors would be clarified with the method described.

Introduction

Substance P, a member of tachykinin family peptides with an amino acid sequence, -Phe-X-Gly-Leu-Met-NH_2 at the C-terminus in common acts as a sensory-neurotransmitter in the central and peripheral nervous system, by interacting with specific receptors present on the membrane surface. The receptors for tachykinins are currently classified into three subtypes of neurokinin $(NK)_1$, NK_2 and NK_3, presumably recognizing substance P, neurokinin A and neurokinin B, respectively[1,2], as the molecular structures were recently determined[3,4,5]. ^{125}I-Bolton-Hunter substance P, a compound of the peptide of which the Lys^3 residue is conjugated with monoiodinated ^{125}I-Bolton-Hunter reagent[6] was found to be a radioligand and was used to detect specific receptor for substance P in the rat parotid cell and brain cortex membranes[7,8]. Accumulating evidence revealed that the compound is a suitable radioligand in the receptor autoradiographic techniques used to detect specific receptor of substance P[9,10,11]. Among three subtypes of tachykinin receptors, NK_1 receptor seems to be predominantly recognized by ^{125}I-Bolton-Hunter substance P[1,12,13].

This chapter describes our quantitative receptor autoradiographic method for specific ^{125}I-Bolton-Hunter substance P binding sites, functional sites of tachykinins[14,15,16,17]. As we previously found in the case of ^{125}I-[Sar^1]-angiotensin II[18], based on the original technique for benzodiazepine receptors with ^{3}H-ligands[19], the method with ^{125}I-ligands coupled to computerized microdensitometry, and a comparison with ^{125}I-standards makes feasible the quantitative determination and complete characterization of neuropeptides receptors in tissues from a single animal. The techniques have advantages over receptor binding assays with partially purified membrane preparations, including precise anatomical localization and a several fold increase in sensitivity. The method with ^{125}I-ligands also surpasses that with ^{3}H-ligands in quantitation, since tissue quenching of radioisotope energy of ^{3}H is linked to the failure to attain precise quantitations of receptors in myelin-rich areas such as brain white matter[20,21]. Furthermore, as the use of ^{125}I-ligands, the final results can be obtained within a week of the binding experiments and rapid data collection is feasible.

Basic Methodology

Preparation of ^{125}I-standards

1. Add known amounts of increasing concentrations of ^{125}I-labeled compounds to rat brain tissue aliquots previously ground to a paste in a centrifuge tube.
2. Homogenize with a polytron homogenizer to mix thoroughly.
3. Transfer homogenate to a small tube, and then centrifuge at 3000 rpm for 10 min to degass.
4. Freeze the tube on dry ice, and remove frozen paste from the tube.
5. Cut 16 μm thick*1 sections in a cryostat at −16℃, and thaw-mount the sections onto subbed glass slides*2.
6. Desiccate the sections under vacuum at 4℃.
7. Store the standard sections in a desiccator.
8. Sets of standards obtained from consecutive sections are used for determination of protein concentrations

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and for radioactivity.

The method was originally described for ^{3}H-standards by Unnerstall et al.[19] The ^{125}I-standards made of polymer are available from Amersham Laboratories, England. When the concentration of binding sites have to be calculated as an amount of radioligand specifically bound (i.e., fmol) per mg protein, it is necessary to convert the data expressed as fmol/mg weight to fmol/mg protein, by comparing the standard curves obtained from commercially available polymer standards with those from the standards, as described[22].

*1 Thickness of the standards should be the same as of tissues sections.

*2 Glass slides are soaked in alconox solution, removed from the solution and boiled gently in deionized water for 1 hr, then washed three times and soaked in 80% alcohol overnight. The slides were soaked overnight in deionized water and dipped in a freshly made gelatin subbing solution (2.5 g gelatin powder and 250 mg chromium sulfate in 500 ml distilled water), dried overnight in a 37℃ oven, and stored in dust-free slide boxes.

Determination of the characteristics curve of the film

Prior to the quantitation of binding sites specifically labeled by ^{125}I-ligands, the characteristic curve of the film used has to be determined from the optical densities obtained with different exposure times of the same set of ^{125}I-standards. Our data obtained using ^{3}H-Ultrofilm is shown in the left side of Fig. 1[18]. The curve was generated by the semilog plot of the optical densities read from the filmvs the product of the amount of radioactivity present in each standard and the exposure time. As shown in Fig. 1, the characteristic curve of ^{125}I-standards and ^{3}H-Ultrofilm was similar to that of ^{3}H-standards. The slope of the curve gives a measure of the photographic contrast. At an optical density of 0.2 unit or less, the changes in the slope as a function of exposure time are very small, and discrimination between different values is difficult. At an optical density of 0.2 unit or less, the changes in the slope as a function of exposure time are very small, and discrimination between different values is difficult. At optical densities greater than 1.6 units, the film rapidly saturates and no useful data can be obtained. A linear relationship ($\gamma = 0.934$) exists between optical density and dpm/pg of protein x time of exposure for values between 0.3 and 1.6 optical density units. In this case, the contrast is high between different optical densities values, making it easy to discriminate between small differences in concentration of radioactivity, i.e. between different concentrations of binding sites for a particular structure. With this information, the exposure time which produces

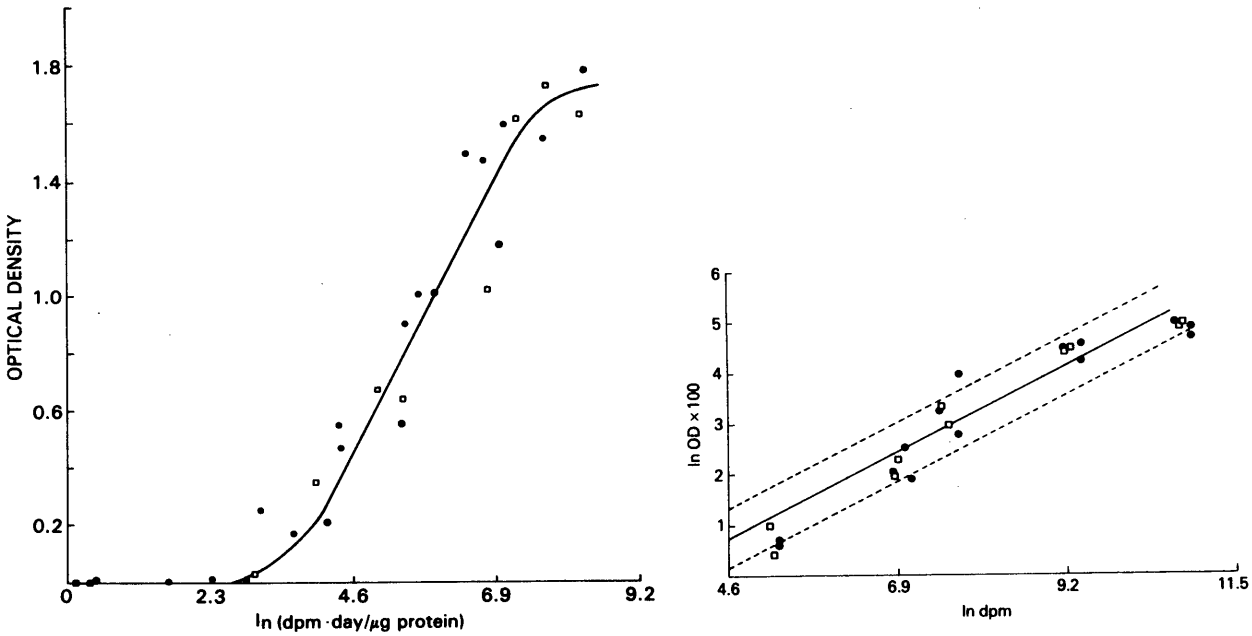

Fig. 1. Quantitative autoradiographic technique for ^{125}I-ligands. Right Panel : A ln-ln plot of O.D. vs. radioactivity (dpm/μg protein) in standards. Exposure times were 1 (□) and 2 (●) days. Each point represents the average of three optical density readings from autoradiographs generated from triplicate standard curves and prepared for each film utilized for the quantitation of A II receptors. Dashed lines represent the stndard error of estimate of X from Y. The general equation of the straight line was, for 24 pairs of data : $Y = 0.757\ X = -2.76$; $g = 0.84$; $F = 68.4$ ($P<0.001$). Left panel : Characteristic curve of [^{3}H] Ultrofilm with ^{125}I-standards. Optical densities are plotted as a function of ln of dpm/μg protein x exposure time. Each point represents the average of three optical density readings from autoradiograms generated from triplicate standard curves. [Reproduced with permission from Ref. 23.]

the optimal contrast can be estimated for a particular tissue section. It is therefore desirable, in preliminary experiments, to expose the labeled tissue sections for varying lengths of time to determine the adequate conditions for optimum contrast (optical densities between 0.6 and 1.4 units for each particular structure).

^{125}I-standard curves

Based on the data from the characteristics curves determined as described above, ^{125}I-standard curves can be obtained. The curve shown in the right side of Fig. 1 was calculated by measuring the optical densities of the autoradiogram (right side in Fig. 2) obtained from each set of standards contained concentration of radioactivity/ μg protein varying from 9.17 to 1008 dpm, after 4 days exposure to ^{3}H-Ultrofilm. There was a linear relationship between the In of optical densities and the In of the concentration of radioactivity at any exposure of ^{3}H-Ultrofilm to the standards. As shown in Fig. 2, tissue sections labeled in vitro by ^{125}I-ligands were placed together with ^{125}I-standards in a X-ray cassettes and opposed against the film. *3 The molar quantities of ligand bound/ mg protein of tissue were determined by interpolating the optical density in the straight line obtained from the In-In standard curve and correcting according to the equation : [mmol/Ci (spec. act.)]x [1 Ci/2.22 x 10^{12} dpm] x 10^{3} = fmol/mg protein.

*3 As the method with ^{125}I-ligands led to the first quantitation of ^{125}I-[Sar^{1}]-angiotensin II binding sites[18], the figure of specific angiotensin II binding sites in the rat adrenal gland previously published was presented, as an example[23].

In Vitro Labeling of Tissue Sections with ^{125}I-Bolton-Hunter Substance P

Tissue preparation

Animal tissues rapidly removed or human tissues obtained at autopsy are frozen by immersion in isopentane at −30℃. Frozen tissues can be stored in air-tight bags at −80℃. Frozen, 16 μm-thick sections are cut in a cryostat at −14℃, thaw-mounted onto glass slides, and dried under vacuum in cold room at 4℃ overnight. After tissue sections are dried, slides are transferred to boxes in the cold room, and the boxes are hermetically sealed.

Preincubation

When the slides are transferred from boxes to stainless steel racks, care must be taken to prevent exposure to humidity. The boxes are left at room temperature, and

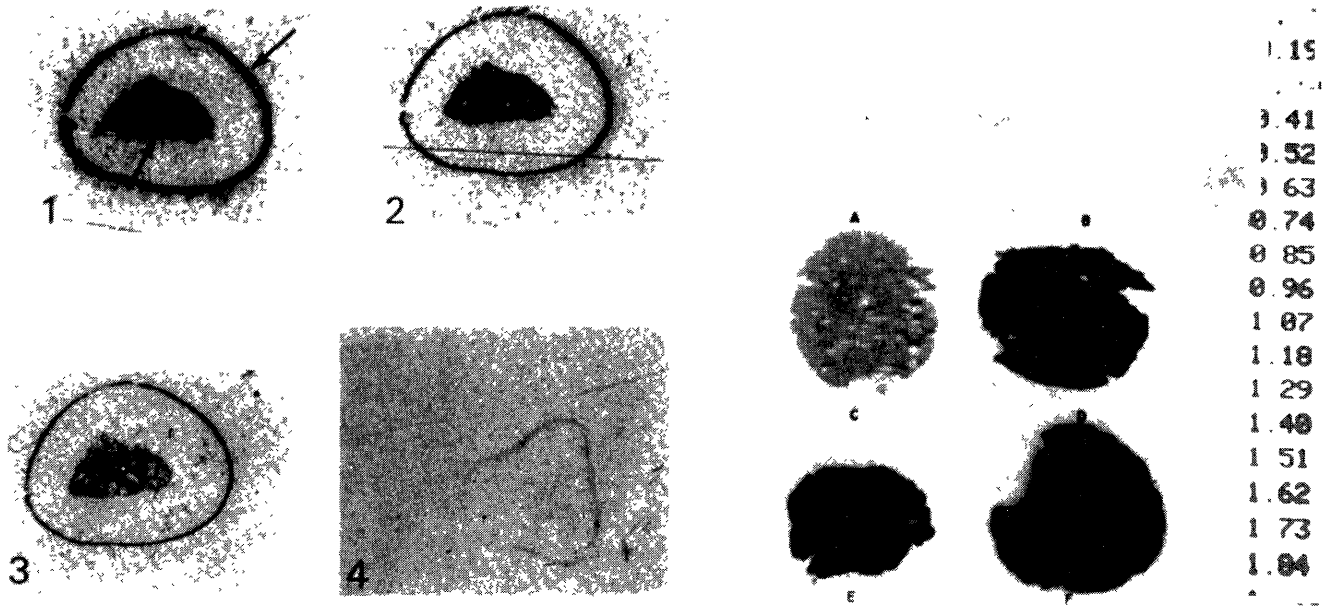

Fig. 2. Angiotensin II receptors in rat adrenal gland. Left panel : 1, 2 and 3. Rat adrenal gland (16 μm sections) incubated with 5.0, 1.25 and 0.32 nM ^{125}I-[Sar^{1}]-A II. Arrows point to zona glomerulosa and to adrenal medulla. 4. Adjacent section incubated with 5 nM ^{125}I-[Sar1]-AII and 25 μM unlabeled A II. Right panel : Autoradiographic image of ^{125}I-standards with computerized densitometry. Each section contains a different amount of radioactivity per mg of protein. In dpm : A, 9.17 ; B, 20.8 ; C, 72.8 D, 200.5 ; E, 536.4 ; F, 1008.0. [Reproduced with permission from Ref. 23.]

opened to transfer the slides to stainless steel racks.

Tissue sections dried are first preincubated for 5 min at room temperature in 50 mM Tris-Hcl buffer, containing 0.2 mg/ml bovine serum albumin (BSA) and 0.005% (vol/vol) polyethylenimine, and then preincubated for 10 min in the same buffer without polyethylenimine[14]. As described by Mantyh et al.[24], polyethylenimine at the concentration of 0.005% prohibits the radioligands to bind nonspecifically to the glass.

Incubation

After preincubation, tissue sections are labeled in vitro with ^{125}I-Bolton-Hunter substance P. The sections on slides are incubated at room temperature for 90 min with the radioligand in an incubation box containing incubation buffer, 50 mM Tris-Hcl buffer, pH 7.4, 0.2 mg/ml BSA, 40 μg/ml bacitracin, 4 μg/ml leupeptn, 50 μg/ml chymostatin, and 5 mM $MnCl_2$ [14,24]. The binding sites can be characterized using consecutive tissue sections. For example, in the equilibrium binding study, consecutive tissue sections are incubated in the presence of increasing concentrations of ^{125}I-Bolton-Hunter substance P, and a fixed amount of unlabeled substance P. In displacement experiments to examine specificity of the binding sites, the sections are incubated in the presence of increasing concentrations of unlabeled tachykinins and related peptides, and a fixed amount of ^{125}I-Bolton-Hunter substance P.

^{125}I-Autoradiography

After incubation, the slides are washed four times for 30 sec each in ice-cold 50 mM Tris-HCl buffer and dried under a stream of air. If necessary, the tissue sections on slides are dried in an air-tight desiccator overnight. The slides are placed in X-ray film cassettes along with ^{125}I-standards, and opposed against ^{3}H-Ultrofilm (LKB industries) or Hyperfilm-^{3}H (Amersham) at room temperature. After adequate exposure, the films are developed at 20℃ for 4 min with undiluted D19 Kodak developer. A complete set of ^{125}I-standards has to be processed with every cassettes and developed with every film.

Data analysis

Optical densities are measured by computerized microdensitomery in both ^{125}I-standards and specific areas of tissue sections from each film. After determination of the

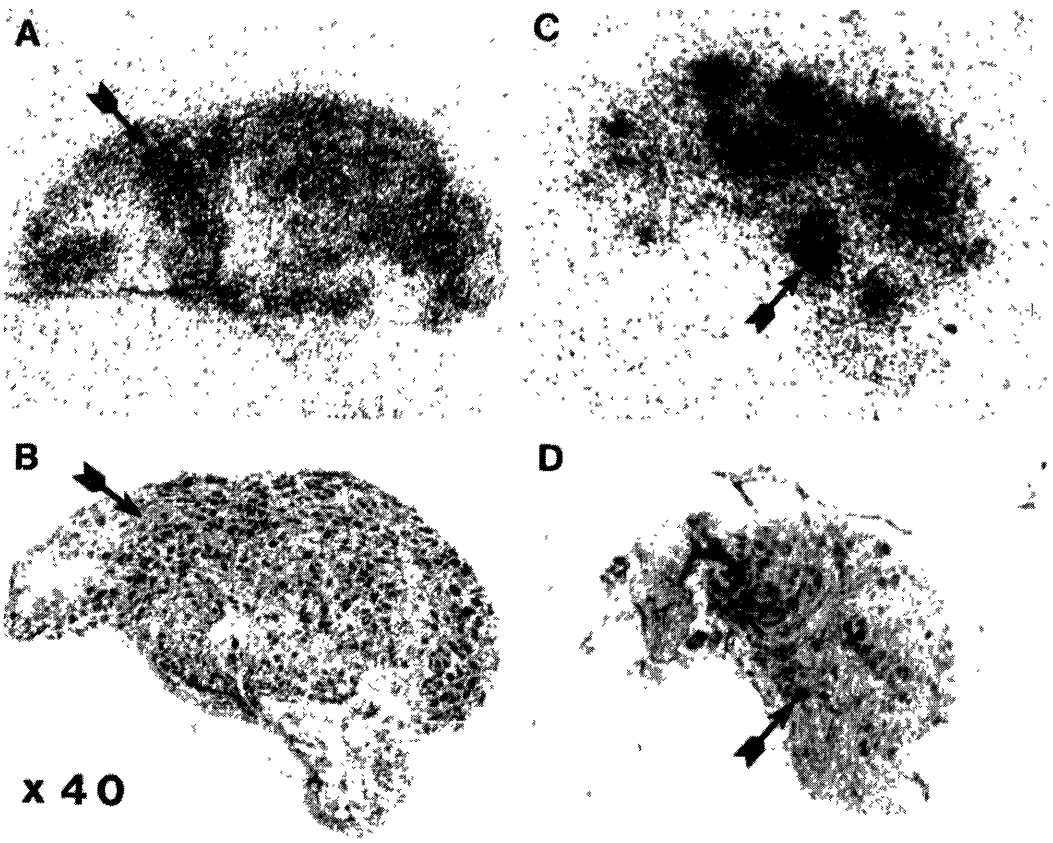

Fig. 3. Receptor autoradiographic evidence of ^{125}I-Bolton-Hunter substance P binding sites in rat celiac sympathetic ganglia, exposure time 12 h (A, B) and superior cervical sympathetic ganglia, exposure time 6 days (C, D). B and D are sections adjacent to A and C, respectively, stained with Toluidine blue. Arrows point to clusters of substance P binding sites (A, C) and to clusters of ganglion cells (B, D). [Reproduced with permission from Ref. 14.]

standard curves [In optical densities x 100 vs In disintegrations per minute (dpm) of standards], as described in the section of Basic Methodology, the optical densities of the tissue areas studied are interpolated in the straight line to obtain the corresponding dpm bound to the tissue[18]. Results are corrected for the decay of ^{125}I. Calculations of the molar quantities of ligand bound to the tissue, saturation curves, and Scatchard analysis are performed by the use of the LIGAND computer program[25,26], followed by correction for the protein content of the standards.

^{125}I-Bolton-Hunter substance P binding sites

Using the receptor autoradiographic technique, specific binding sites for ^{125}I-Bolton-Hunter substance P, functional sites of NK_1 receptor predominantly recognizing substance P have been assayed in the central nervous system and peripheral organs. Taking advantage of this assay method, the binding sites in very small tissues such as rat sympathetic ganglia were localized and quantitated[14]. As shown in Fig. 3, nodular and heterogeneous distributions of the binding sites in the superior cervical sympathetic ganglion (A in the figure) and celiac-superior mesenteric sympathetic ganglion (C) were detected. When compared with the adjacent sections stained by Toluidine blue, ^{125}I-Bolton-Hunter substance P binding sites were located in the areas of high density of principal ganglion cells (cell bodies of the postganglionic noradrenergic neurons) both in the ganglia (B and D). The superior and celiac ganglia had a single class of binding sites with Kds (dissociation constants) of 0.49 nM and 0.58 nM, respectively. As nerve endings of primary sensory neurons containing substance P originating from the dorsal root ganglia have been seen surrounding principal ganglion cells[27], the existence of specific ^{125}I-Bolton-Hunter substance P binding sites supports the excitatory modulation of substance P on the ganglionic transmission between the pre- and postganglionic sympathetic neurons.

We also detected the binding sites in the human skin tissues, pad skin obtained from a thumb excised from a 12-month-old boy with polydactylia[17]. The highest density of ^{125}I-Bolton-Hunter substance P binding sites was noted

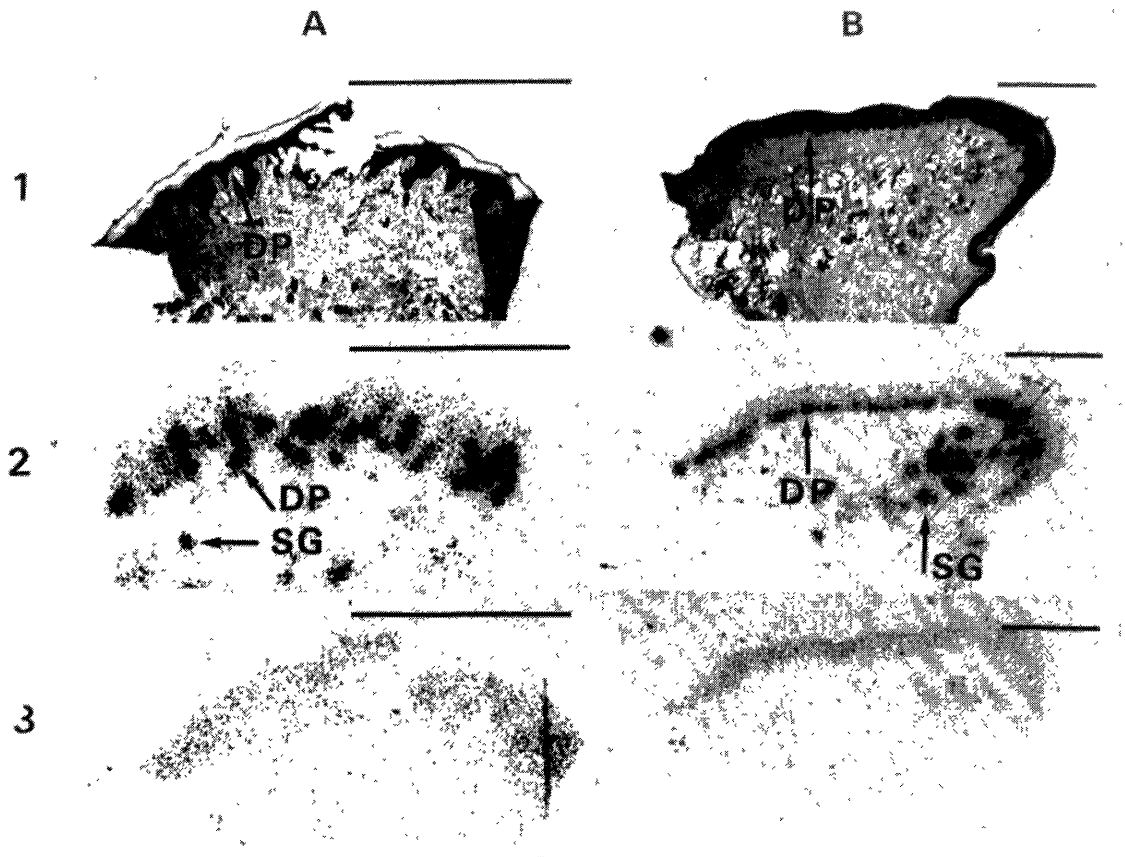

Fig. 4. Receptor autoradiographic evidence of ^{125}I-Bolton-Hunter substance P binding sites in the finger pad skin obtained from a thumb excised from a 12-month-old boy with polydactylia (A), and from a rat paw pad skin (B). Consecutive, 16 μm thick tissue sections were incubated with 100 pM of the radio-labeled ligand, without (middle panel) and with 1.0 μM of unlabeled substance P for non-specific binding (lower panel). Dried sections were exposed to ^{3}H-Ultrofilm for 4 days. Adjacent sections were stained with hematoxylin-eosin to observe the anatomy (upper panel). DP, dermal papilla ; SG, sweat gland. Bar = 1.0 mm. [Reproduced with permission from Ref. 17.]

in the dermal papilla and sweat gland (Fig. 4). The binding to the dermal papilla was of single and high affinity with a Kd of 744 pM. Of particular interest is the observation that the highest density of the binding sites was in the dermal papilla, an area which contains substance P-containing free ending, in addition to the nerve endings innervating capillaries[28]. In this study, light microscopic examination of emulsion autoradiography revealed that the ^{125}I-Bolton-Hunter substance P binding in the dermal papilla of the human thumb pad skin did not only over the vascular endothelial cells, but also in the areas devoid of vascular component (data not shown). As the free nerve endings are nociceptors related to the transmission of pain[29], evidence for the existence of the binding sites in an area of the dermal papilla which lacks the vascular component seems pertinent to the proposal that the binding sites are presynaptic receptors on the free nerve endings and may relate to pain transmission.

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