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# Immunochemical detection of photoaffinity-labelled capsaicin-binding proteins from sensory neurons

John N. Wood, Christopher Walpole, Iain F. James, Andy Dray and Paul R. Coote

*The Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6 BN, UK*

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Capsaicin is a plant neurotoxin which depolarises a subset of mammalian sensory neurons. A photoaffinity probe (4-azidophenylpropionamide) with capsaicin-like agonist activity ($EC_{50}$ 5 $\mu$M) has been used to covalently label rat and chick sensory neurons in culture, as well as membrane preparations from both neurons and other tissues. Dorsal root ganglion cell specific capsaicin-binding proteins, including a major band of apparent molecular mass 58 000, have been identified by means of Western blotting, using a specific anti-capsaicin antiserum characterised by radioimmunoassay with a large range of capsaicin congeners. Using the same radioimmunoassay, no endogenous capsaicin-like immunoreactive material in normal or inflamed tissue has, however, been detected.

Capsaicin; Sensory neuron; Receptor; Photo-affinity labelling

## 1. INTRODUCTION

Capsaicin (8-methyl *N*-vanillyl 6-nonenamide) is the principal pungent principle of red pepper plants. This compound exerts excitatory, desensitising and toxic actions on a subset of sensory neurons, including the polymodal nociceptors that are activated by a range of noxious stimuli. Capsaicin depolarises sensory neurons [1,2] increasing sodium, potassium and calcium fluxes in susceptible cells [3] probably through a direct interaction at the plasma membrane [4] rather than through any second messenger mediated events [5]. The effects of capsaicin are both cell and species specific. Structure-function analysis with a range of capsaicin congeners has led to a model of a proposed capsaicin receptor [6], and irreversible agonism demonstrated by means of capsaicin-like photoaffinity probes, supports the notion that a specific capsaicin binding site exists [7]. The lipophilicity and relatively low efficacy of capsaicin ($EC_{50}$ 0.07 $\mu$M for most actions) [3] has made direct binding studies difficult, and no direct identification of capsaicin-binding molecules has yet been made. It is thus unclear if the capsaicin-binding site corresponds to a physiologically relevant receptor, for which, by analogy with the opioid peptides, there are capsaicin-like endogenous ligands. One approach to defining the capsaicin-binding site is to use photoactivatable affinity labels with capsaicin-like activity, to covalently tag capsaicin-binding molecules. We have therefore used a capsaicin derivative containing a photoactivatable azide group, which can be detected with a polyclonal anti-capsaicin antiserum characterised in a competition radioimmunoassay with a large number of capsaicin congeners. Photoaffinity labelling of rat and chick sensory neurons in culture, and of membrane extracts of bovine dorsal root ganglia (DRG) and other tissues, followed by Western blotting, reveal a number of capsaicin-binding proteins. Radioimmunoassay of normal and inflamed tissue extracts however, failed to provide evidence for the existence of endogenous capsaicin-like immunoreactive material.

*Correspondence address:* J.N. Wood, The Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN, UK

## 2. MATERIALS AND METHODS

### 2.1. *Anti-capsaicin antisera*

*N*-(3-methoxy-4-hydroxy benzyl) 6'-aminohexamine hydrochloride was synthesised from BOC-$\epsilon$-aminocaproic acid-N-hydroxysuccinimide ester (Bachem A15650) and *O*-2-ethoxyethylvanillylamine (Sandoz Kilolab, KL 3342 (synthesis described in European patent application 82200796.9) Procter and Gamble 1982). This compound was covalently coupled to thyroglobulin with glutaraldehyde [8] and half-lop rabbits were immunised with the conjugate emulsified in Freunds complete adjuvant (Difco). At monthly intervals, rabbits were further immunised with the complex emulsified in Freunds incomplete adjuvant. Rabbit bleeds were tested in a competition radioimmunoassay (see below).

### 2.2. *Photoaffinity labelling*

The photoaffinity probe *N*-(vanillyl)-4-azidophenylpropionamide (APP) was synthesised as follows; The *N*-hydroxysuccinimide ester of 4-hydroxyphenylpropionic acid was made by dicyclohexyl-carbodiimide coupling of the acid with *N*-hydroxysuccinimide in dimethylformamide. The purified ester was then stirred at room temperature with vanillylamine for 12 h before purification of the product under subdued light.

Spectral and analytical data were consistent with the given structure. Capsaicin induced $^{45}Ca^{2+}$ accumulation into sensory neurons

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was measured as described [3]. Briefly, Terasaki plates containing about $10^3$ sensory neurons/well were incubated with varying concentrations of APP in 10 μl calcium-free HEPES-buffered Hanks solution (pH 7.4), rinsed and irradiated for 90 s at 254 nM with a light source (Gallenkamp UVP) positioned 1.5 cm from the cells (1250 $\mu W/cm^2$), leading to complete activation of the nitrene. After washing, 10 μl HEPES-buffered Hanks solution containing 10 μCi/ml $^{45}Ca^{2+}$ (1 mCi/μMol) was applied to the Terasaki wells, and calcium accumulation over a 10 min period was measured in control and treated cultures. Rat and chick DRG neurons for Western blot analysis were grown on coverslips (40000 neurons/coverslip), incubated with APP and additions, rinsed and irradiated as described above. The cells were then treated with Laemmli sample buffer (100 μl) containing 1% mercaptoethanol, and analysed (25 μg protein/track) by SDS-PAGE on 10% gels [9]. Gels were electroblotted and immunoreactive material was detected by incubation with 1:400 dilution of anti-capsaicin rabbit antiserum followed by [$^{125}$I]anti-rabbit serum (Amersham), and autoradiography. A hyper-immune anti-cAMP rabbit serum was used as a control serum. Preparations enriched in cell membranes from rat and cow DRG were prepared by homogenisation in 10 mM HEPES pH 7.4 at 4°C. The low speed spin pellet (1000 g × 10 min) was discarded and material pelleted by centrifugation (18000 rpm, Sorval SS34, rotor, 4°C, 1 h) was retained. Membranes were resuspended in PBS (5 mg/ml), incubated with a range of concentrations of APP in 96-well plates, and irradiated as described above. Laemmli sample buffer (5 volumes) was then added to the membrane samples which were analysed by Western blotting as described above.

### 2.3. *Radioimmunoassay*

Capsaicin was reductively tritiated to [di-$^3$H]capsaicin (8-methyl *N*-vanillyl [6,7-$^3H_2$]nonanamide) of specific activity 50 Ci/mMol (Amersham) [7]. A competition binding assay was set up using a 1:200 dilution of a single rabbit bleed incubated with 20,000 dpm [di-$^3$H]-capsaicin in phosphate buffered saline (PBS, pH 7.4) containing 0.1% BSA (Sigma) and capsaicin standards or unknown samples. Incubations were carried out at 4°C overnight, and bound material was separated by incubation with cellulose coupled anti-rabbit antisera (Saccel, Wellcome) using a similar protocol to that described for cyclic nucleotide radioimmunoassay [5]. All capsaicin-related congeners used in the radioimmunoassay were fully characterised by $^1$H-NMR, mass spectrometry and elemental analysis (C,H,N) and were at least 98% pure by TLC and HPLC (C. Walpole in preparation). Rat tissues were homogenised using a polytron. The solutions used for homogenisation were PBS containing 0.1% NP40 (BDH); 10 mM HEPES pH 7.4 containing 1 μM A23187 (Sigma); 0.1 M HCl followed by neutralisation with NaOH and assay in PBS. Homogenates were centrifuged at 10000 rpm in a Sorval SS34 rotor, supernatants serially diluted in PBS and protein concentrations then measured [10].

## 3. RESULTS

A range of capsaicin congeners were tested in a competition radioimmunoassay using [di-$^3$H]capsaicin (Fig. 1). The structural requirements for recognition by the antiserum were precise, and are compared with those required for activity in a calcium accumulation assay in Table I. The presence of a 3-methoxy group (compound O) and a 4-hydroxyl function (compound N) is obligatory. Reversing the amide bond (compound C) leads to a small loss in immunoreactivity, and has little affect on agonism. Substituting the bond with a sulfonamide (D) a hydroxyamide (F), thiourea (G), N-cyanoguanidine (H), ester (I) thioester (J), or a reversed ester (K) leads to a progressive loss in immunoreactivity, which shows less correlation with agonist activity as

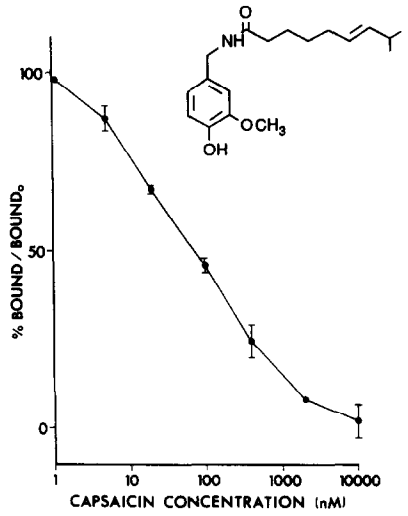


Fig. 1. Radioimmunoassay of capsaicin using [di-$^3$H]capsaicin. The percentage of bound ligand is plotted as a function of capsaicin concentration. The structure of capsaicin is shown in insert. The radiolabelled ligand has [$^3$H] incorporated into the 6,7 double bond position as described in the text.

measured by calcium accumulation. The presence of an aliphatic side chain appears to be necessary, because vanillylamine, eugenol and 3-methoxytyramine all lack immunoreactivity. However both octyl (B) and phenethyl (A) side chains are recognised equivalently. Related catecholamines were little recognised in the assay, consistent with the requirement for a hydrophobic side chain, the structure of which need not be well-defined. Thus structural features involved in immunoreactivity and agonist potency show substantial similarities, although there are some anomalies within the amide bond region.

Having established the specificity of the antiserum, the sensitivity of detection of capsaicin-derived photoaffinity probes was assessed on dot blots of photoaffinity labelled albumin. 4-azidophenylpropionamide (APP) was a reasonably potent capsaicin-like agonist, ($EC_{50}$ 5.5 μM for $^{45}Ca^{2+}$ uptake) which could be detected at a level of 50 fmol on dot blots (not shown). Such sensitivity allows ng quantities of a protein of 60,000 molecular weight covalently bound by a single molecule of APP to be detected.

When APP was incubated with sensory neuron cultures and irradiated at 254 nm with a UV-source for 90 s, irreversible agonism could be demonstrated by comparing $^{45}Ca^{2+}$ accumulation into extensively washed control and irradiated cultures (Fig. 2). The photoaffinity label therefore binds at a site which is closely apposed to that involved in agonism.

Cultures of neonatal rat DRG neurons were preincubated with a range of concentrations of APP (1–100 μM) in low calcium buffer, and irradiated in the presence or absence of 30 μM capsaicin, then washed and analysed by SDS-PAGE. In addition, membrane

Table I

A comparison of the immunoreactivity and agonist potency of a range of capsaicin congeners

| Compound | R3 | R4 | Bond | Side-chain | $IC_{50}$ | $EC_{50}$ calcium ($\mu$M) |
|---|---|---|---|---|---|---|
| Capsaicin | OMe | OH | $CH_2NHCO$ | $(CH_2)_4CH:CHCHME_2$ | 120nM | 0.2 |
| A | OMe | OH | $CH_2NHCO$ | $CH_2CH_2 \cdot Ph$ | 110nM | 45 |
| B | OMe | OH | $CH_2NHCO$ | $(CH_2)_7Me$[octyl] | 120nM | 0.4 |
| C | OMe | OH | $CH_2CONH$ | octyl | 350nM | 0.13 |
| D | OMe | OH | $CH_2$ NH $SO_2$ | octyl | 600nM | 1.4 |
| E | OMe | OH | $CH_2$ NH CO | $CH:CH \cdot Ph(p\text{-}NO_2)$ | 650nM | 7.0 |
| F | OMe | OH | CH OH CONH | octyl | 14$\mu$M | 1.1 |
| G | OMe | OH | $CH_2$ NHCSNH | octyl | 16$\mu$M | 0.08 |
| H | OMe | OH | $CH_2$ NH(C:NCN) NH | octyl | 130$\mu$M | 3.2 |
| I | OMe | OH | $CH_2$ COO | octyl | 160$\mu$M | 1 |
| J | OMe | OH | $CH_2$ COS | octyl | 250$\mu$M | 2 |
| K | OMe | OH | $CH_2$ OCO | octyl | 900$\mu$M | 9 |
| L | OMe | OH | CONH | octyl | 1.1mM | Inactive |
| M | OMe | H | CONH | octyl | >1.5mM | Inactive |
| N | OMe | H | $CH_2$ NHCO | octyl | >1.5mM | Inactive |
| O | H | OH | $CH_2$ CONH | octyl | >1.5mM | 5 |
| Eugenol | OMe | OH | $CH_2$ CH:$CH_2$ | - | >1.5mM | Inactive |
| 3-Methoxytyramine | OMe | OH | $CH_2$ $CH_2$ $NH_2$ | - | >1.5mM | Inactive |
| VA | OMe | OH | $CH_2$ $NH_2$ | - | >1.5mM | Inactive |

The $IC_{50}$ measured by radioimmunoassay is compared with the $EC_{50}$ for calcium accumulation [3] into sensory neurons evoked by a range of capsaicin congeners, where R3 represents the 3-methoxy group and R4 represents the 4-hydroxyl group on the aromatic ring of capsaicin

extracts of rat liver, cerebellum and DRG, and chick and bovine DRG were prepared and photoaffinity labelled in a similar fashion. Covalently photoaffinity labelled proteins were detected by Western blotting with anti-capsaicin antisera.

A typical experiment is shown in Fig. 3, where 30 $\mu$M APP was incubated with chick DRG, rat DRG, rat liver and rat cerebellar membranes. A range of proteins are labelled by the probe including a major band of apparent molecular weight 58 000 which is DRG specific, together with a DRG-specific minor band of apparent molecular weight 42 000. Coincubation of 30 $\mu$M capsaicin with APP during the incubation and irradiation did not detectably inhibit APP binding to any bands (not shown).

The possibility that capsaicin mimics the action of an endogenous ligand was investigated by analysing extracts of homogenised tissue for the presence of

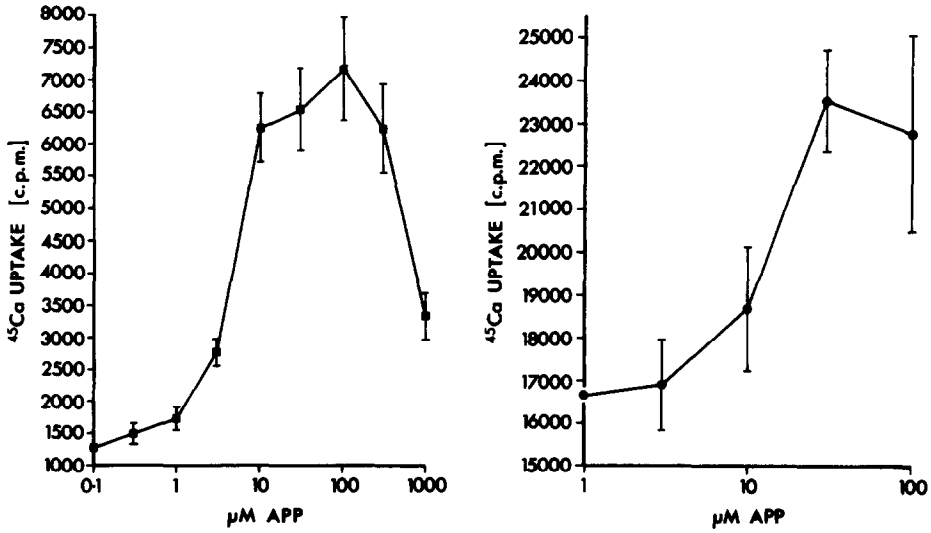


Fig. 2. APP is a capsaicin-like agonist after photoactivation. The accumulation of $^{45}Ca^{2+}$ into DRG neurons in culture as a function of APP concentration is plotted in the left panel for unactivated ligand. Assays were carried out precisely as described [3]. In the right panel, APP was photoactivated, and unbound ligand removed by washing the cells, before addition of 50 $\mu$Ci/ml $^{45}Ca^{2+}$. Control experiments, where cells were preincubated with APP without irradiation, then washed and assayed with capsaicin, showed that background uptake was 16 300 cpm (SE 2310) and 2 $\mu$M capsaicin evoked an uptake of 23 125 cpm (SE 2277) ($n=6$).

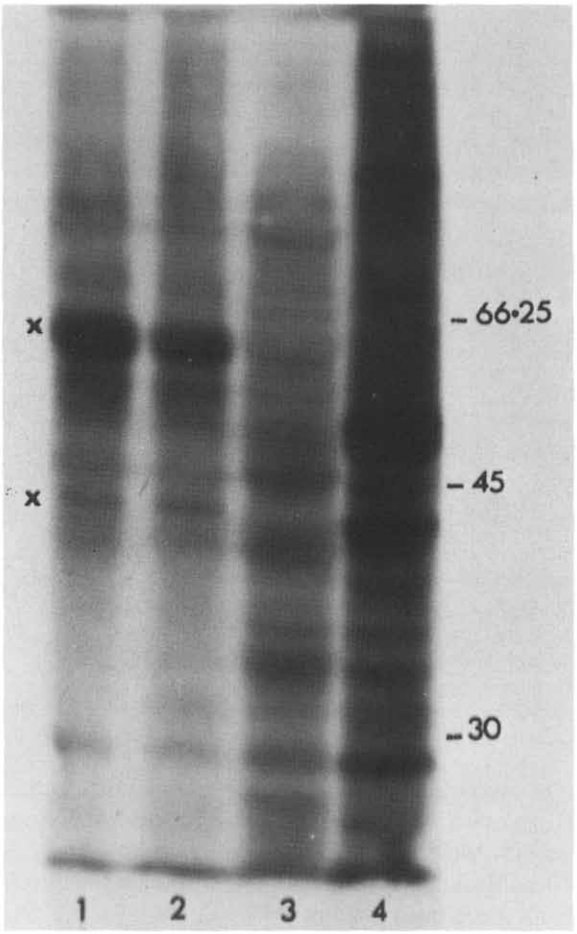


Fig. 3. Immunochemical detection of photoaffinity labelled proteins. Western blot of photoaffinity-labelled tissues separated by SDS-PAGE on a 10% gel. Track 1, Rat DRG membranes (25 μg); 2, chick DRG membranes (25 μg); 3, rat cerebellar membranes (50 μg); 4, rat liver membranes (50 μg). Those bands marked with an asterisk are found in DRG but not other tissues. Molecular weights of markers are given in kDa.

capsaicin-like immunoreactive material by radioimmunoassay. Adult Sprague-Dawley rat tissues assayed were; spinal cord, dorsal root ganglia, whole brain, cerebellum, pituitary, hypothalamus, skeletal muscle, heart, liver and kidney. Normal and inflamed foot pads (50 μl, 20% yeast in saline, injected 2 h before homogenisation) were obtained from Balb/c mice.

Protein concentrations of 10–20 mg/ml were initially used and serially diluted in PBS. A number of homogenisation buffers were used including a solution containing A23187, a divalent cation ionophore that raises intracellular calcium, and may thus induce the release of neuroactive factors [11]. No immunoreactive material could be detected despite a sensitivity of 1 pmol for capsaicin-like immunoreactive material in the radioimmunoassay. The only tissue extract that displaced capsaicin binding was liver, at concentrations higher than 5 mg/ml. It is known that catecholamine metabolism, occurs in the liver, and high concentrations of vanillylamine containing catabolites are present in this tissue [12].

## 4. DISCUSSION

Capsaicin, because of its selective toxic actions has proved to be a useful pharmacological tool in the analysis of sensory neuron function [13]. Present evidence suggests that capsaicin increases cation fluxes by a direct interaction at the plasma membrane [4]. The existence of an operationally defined capsaicin-receptor is not in doubt, but whether such a receptor is physiologically modulated by endogenous ligands that are structurally related to capsaicin is not known.

The existence of an endogenous ligand with capsaicin-like activity seems unlikely, because of the toxic effects of capsaicin [14]. There remains the possibility that a capsaicin-like endogenous mediator with less potent activity than capsaicin exists. It has been difficult to address the problem of capsaicin-like endogenous ligands functionally, because of the broad range of mediators that depolarise sensory neurons. In addition, experiments with antibodies raised against opioid peptides which do not cross-react with plant opioids suggest that any endogenous capsaicin-like ligand might not show similar immunoreactivity to capsaicin [15]. We nonetheless radio-immunoassayed a range of normal and inflammed rat tissue extracts. Only in liver extracts that contain high concentrations of catecholamine breakdown products [12] was any immunoreactive material detected. Such material, because of its distribution, is unlikely to play a role in modulating the activity of sensory neurons.

The rat DRG-specific immunoreactive bands detected on Western blots are likely to correspond to, or be situated close to, capsaicin-binding proteins. Our inability to protect these proteins from labelling by co-incubation of APP with capsaicin does not allow them to be assigned as part of the capsaicin binding site, although the lipophilicity and relatively low affinity of all the reagents used, combined with the long period required for activation of the photoaffinity label may explain our failure to achieve protection. The fact that chick DRG neurons also contain similar molecular weight proteins, despite being insensitive to capsaicin [16], argues against these proteins being the capsaicin binding site of a cation-selective ion channel, although it is possible that a similar molecule may have distinct functions in the two species.

In summary, we can provide no evidence for capsaicin-like immunoreactive material in normal or inflammed tissue, using a radioimmunoassay with a sensitivity of less than 1 pmol for capsaicin. However, we have been able to detect DRG-specific capsaicin binding proteins immunochemically through the use of a photoaffinity probe. The possible relation of these proteins to

the putative capsaicin receptor is unknown. However, the recent development of a binding assay using resiniferatoxin, a high affinity capsaicin-like toxin [17] should eventually resolve this question.

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