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Long-Lasting Agonist Activity Produced by a Capsaicin-like Photoaffinity Probe

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SUMMARY

Capsaicin causes uptake of $^{45}Ca^{2+}$ into a subset of neurons cultured from rat dorsal root ganglia. One aryl azido analogue of capsaicin produced a long-lasting stimulation of calcium uptake after irradiation in contact with cells, but not if samples were kept in the dark. The active compound, like capsaicin, is a 3-methoxy, 4-hydroxy benzylamide. An analogue without substitutions on the benzyl ring and one without the ring were not agonists. This pattern is consistent with known structure-activity relationships for capsaicin analogues. All three aryl azides produced long-lasting inhibition of capsaicin action, which was not totally dependent on irradiation. Long lasting agonist action was not produced if reversible capsaicin agonists were present as protecting ligands during the irradiation step. Potency as protectors correlated well with potency as agonists in the calcium uptake assay. We suggest that the active aryl azide is a photoaffinity label for a specific capsaicin receptor on dorsal root ganglion neurons but the inhibitory effects are not dependent on binding to the receptor.

Capsaicin is a pharmacologically active component of red peppers. Its effects include induction of pain (1–4), a decrease in body temperature (1, 5), a decrease in blood pressure, and changes in heart rate (6). It causes contraction of isolated guinea pig ileum (7, 8) and has effects on isolated guinea pig heart (7, 9). Sensory neurons are depolarized by capsaicin (10–13) and substance P is released in spinal cord (14–16). Capsaicin is also a neurotoxin, causing selective loss of unmyelinated sensory fibers (C fibers) when administered systemically to newborn and adult rats (17–19). Rapid desensitization is a characteristic of capsaicin action, the initial excitatory response being followed by insensitivity to subsequent capsaicin application and cross-desensitization to other noxious stimuli (20, 21). In rats, mice, and humans capsaicin causes an increase in nociceptive thresholds (2, 4, 5, 22), possibly as a result of this type of desensitization. The excitatory, desensitizing, and toxic effects of capsaicin are selective for C fibers and a population of Aδ fibers (see Ref. 23 for a review). Such selectivity means that capsaicin is a useful tool to study the functions and properties of these neurons.

The structure of capsaicin is shown in Fig. 1B. There have been several studies of structure-activity relations (5, 24–27). The phenyl ring is very important; unsubstituted and 3,4-dimethoxy derivatives have much reduced activity compared with the 3-methoxy, 4-hydroxy compound (26). There is some flexibility with the amide bond. The position of the —NH and carbonyl functions can be reversed or the amide can be replaced by an ester without much loss of activity (26). The alkyl chain is important in that its removal leads to loss of activity, but neither the length nor the double bond is critical (26). Some cyclic structures are also allowed in this part of the molecule (24, 26). The selectivity of capsaicin action and the fairly rigid structural requirements for activity suggest that there may be a capsaicin receptor on sensory neurons. A model for such a receptor has been proposed by Szolcsanyi and Jancso-Gabor (26), but the proposal is not yet supported by direct evidence.

In cultures from rat DRG, capsaicin acts on a subpopulation of the neurons, causing depolarization and an influx of calcium ions (11, 28, 29). The effects are selective in that other types of peripheral or central neurons from rat are not affected by capsaicin, nor are primary afferent neurons from chick (28, 29). We have used the increase in $^{45}Ca^{2+}$ uptake into primary cultures of rat DRG neurons as our standard assay of capsaicin activity. This appears to be a specific measure of capsaicin-like effects, because the capsaicin-sensitive channel does not seem to correspond to any of the known ion channels, and no other sensory stimulants that we have tested cause the same level of calcium uptake (29). We have therefore adopted the term

ABBREVIATIONS: DRG, dorsal root ganglia; Ara-C, cytosine-β-D-arabinofuranoside; DHC, dihydrocapsaicin; EGTA, ethylene glycol bis (β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; m.p., melting point; NGF nerve growth factor; PAL 1, *N*-(4-azido-5-iodo-2-methoxybenzoyl)-6′-aminocaproyl vanillylamide; PAL 2, *N*-(4-azido-5-iodo-2-methoxybenzoyl)-6′-aminocaproyl benylamide; PAL 3, *N*-octyl(4-azido-5-iodo-2-methoxy)benzamide; quin2, 2-[(2-bis-[carboxymethyl]amino-5-methyl-phenoxy) methyl] aminoquinoline; VAH, *N*-vanillyl-6-aminohexamide; VBT, *N*-vanillyl-*N*′-butylthiourea; VOS, *N*-vanillyl-octylsulphonamide; VOT, *N*-vanillyl-*N*′-octylthiourea; VPP, *N*-vanillyl-3-phenylpropionamide.

"capsaicin agonist" to describe compounds with capsaicin-like actions. The cultured DRG cells have several of the properties of polymodal nociceptors *in vivo*. The capsaicin-sensitive cells (about 50% of the neurons in our cultures) are relatively small, do not stain with the anti-neurofilament monoclonal antibody RT-97, and are easily desensitized to capsaicin (29–31).

We now present evidence for a specific saturable binding site, which recognizes the substituted phenyl ring in capsaicin and mediates the agonist effects in the calcium uptake assay. We measured long-lasting effects of three different aryl azido capsaicin analogues (Fig. 1A) after irradiation with UV light. One of these produced long-lasting agonism only after irradiation in contact with DRG neurons, but not if samples were kept in the dark. The active compound was a 3-methoxy, 4-hydroxy benzylamide. One compound with an unsubstituted ring and one without this ring were not agonists. The long-lasting agonism was not produced if reversible capsaicin agonists were present during irradiation. All the aryl azides produced long-lasting inhibition of capsaicin action, which was not totally dependent on irradiation. We suggest that the active aryl azide is a photoaffinity label for a specific capsaicin receptor but the inhibitory effects are not dependent on binding to the receptor.

Materials and Methods

All chemicals were AnalaR grade from BDH Ltd., Dagenham, Essex, England, except for those listed below. *t*-Butyloxycarbonyl-ε-amino caproic acid *N*-hydroxysuccinimide ester was from Bachem, Bubendorf, Switzerland, and 4-amino salicylic acid from Aldrich Chemical Co. Ltd., Gillingham, Dorset, England. $^{45}CaCl_2$ was from Amersham International plc, Aylesbury, Buckinghamshire, England. Capsaicin (approximately 90%), Ara-C, poly-*D*-lysine (molecular weight, 130,000) deoxyribonuclease (type I from bovine pancreas), HEPES, and quin2 were from Sigma Chemical Co. Ltd., Poole, Dorset, England. Trypsin was from Cooper Biomedical Inc., Malvern, PA, and collagenase from Boehringer Mannheim GmbH, West Germany. Ham's F-14 medium, horse serum, and Hanks' balanced salt solutions were from GIBCO Ltd, Paisley, Scotland, and Terasaki plates from Nunc, Uxbridge, Middlesex, England. Nerve growth factor was kindly supplied by Dr. R. M. Lindsay, Department of Cell Biology, Sandoz Institute, London, England.

Synthesis of Capsaicin Analogues

Agonists and protectors. DHC was made by catalytic reduction of capsaicin in methanol, using 5% Pd on charcoal as catalyst. The product was recrystallized from 100–120 petroleum ether/ethyl acetate to give colorless crystals (yield, 71% m.p., 62–64°; literature value, 65°). [^{3}H]DHC was produced by Amersham International plc in the same way and purified in our laboratory by high performance liquid chromatography on a Waters C_{18} μ-Bondapak column with a gradient from 70 to 100% methanol in water. Specific activity was about 60 Ci/mmol.

VOT and VBT were synthesized from vanillylamine and the appropriate alkyl isothiocyanate in dimethylformamide in the presence of NaOH by the method outlined in European Patent Application 82200796.9 (Procter and Gamble Company, 1982). VOT was recrystallized from 100–120 petroleum ether/ethyl acetate to give colorless crystals (yield, 50%; m.p. 97–100°; literature value, 100–101°). VBT was recrystallized from diisopropyl ether to give colorless crystals (yield, 20%; m.p., 103–107°).

VPP was synthesized by the condensation of phenylpropionylchloride with *O*-2-ethoxyethyl-protected vanillylamine (described in the above-mentioned Procter and Gamble patent), in ethyl acetate, in the presence of triethylamine. Deprotection was by treatment with 1 M HCl in tetrahydrofuran and the final product was recrystallized from 100–120 petroleum ether/ethyl acetate to give colorless crystals (overall yield, 26%; m.p., 89–92°; literature value, 80–83°). VOS was made in the same way by condensation of the protected vanillylamine with octane sulfonyl chloride. The product was recrystallized from diethyl ether/cyclohexane to give colorless crystals (68% yield; m.p., 79–81°; literature value not reported).

To make VAH, *O*-2-ethoxyethyl vanillylamine was reacted at room temperature with *t*-butyloxycarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester in dimethylformamide. Protecting groups were removed sequentially, by treatment first with 1 M HCl in tetrahydrofuran and then with 50% trifluoroacetic acid in CH_2Cl_2 to give the trifluoroacetate salt of VAH as a colorless oil (overall yield, 68%).

Photoaffinity labels. 4-Azido-5-iodo-2-methoxybenzoic acid *N*-hydroxysuccinimide ester was an intermediate in the synthesis of all three photoaffinity labels. It was synthesized from 4-aminosalicylic acid in six steps: 1) production of the methyl ester by treatment with sulfuric acid in methanol, to protect the carboxyl group; 2) diazotization and treatment with NaN_3 to make the aryl azide; 3) iodination with chloramine T and NaI; 4) methylation of the phenol with methyl iodide in the presence of K_2CO_3; 5) deprotection of the carboxyl group with NaOH in aqueous methanol; 6) production of the active ester by reaction with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide.

Condensation of this intermediate in dimethylformamide with VAH trifluoroacetate in the presence of 1.1 equivalents of triethylamine gave PAL 1 (yield, 38%; m.p., 133–137°; all photoaffinity labels were recrystallized from 100–120 petroleum ether/ethyl acetate). Condensation of the intermediate with octylamine gave PAL 3 (yield, 29%; m.p., 120–121°); condensation with the unsubstituted analogue of VAH (synthesized from benzylamine instead of *O*-protected vanillylamine) gave PAL 2 (yield, 40%; m.p., 120–121°).

Compounds were characterized by proton nuclear magnetic resonance, infrared spectroscopy, mass spectrometry, and elemental analysis. In all cases spectral data were consistent with the assigned structure. So were the analytical data for all compounds except VAH, for which we could not obtain an elemental analysis because it was a hygroscopic oil. VAH was pure by high performance liquid chromatography (Waters C_{18} μ-Bondapak column, gradient from 10 to 50% acetonitrile in 0.1% aqueous trifluoroacetic acid).

Detailed protocols for the synthesis of any of these compounds are available on request. We will supply the compounds themselves as long as our present stocks last.

Preparation of DRG Cultures

Primary cultures of DRG were prepared as described (29). Briefly, DRG were removed from all spinal levels of newborn Sprague-Dawley rats and collected in Ham's Nutrient Mixture F-14 (with L-glutamine, without sodium bicarbonate, supplemented with 10% horse serum and 200 ng/ml NGF). Cells were dissociated by incubating first in 0.125% collagenase at 37° for 35 min, followed by 0.25% trypsin for 30 min and gentle trituration in F-14 medium (containing 10% horse serum and 90 μg/ml deoxyribonuclease) with a fire-polished Pasteur pipette. They were then passed through a 90-μm filter, spun down, and resuspended in F-14 with 10% horse serum, 200 ng/ml NGF, 10 μM Ara-C, and 30% conditioned medium (made by passage of C6 rat glioma cells in F-14 + 10% horse serum). Cells were then plated onto poly-D-lysine-coated Terasaki plates at a density of about 1000 neurons/well, allowed to adhere overnight, and then fed with F-14 supplemented with horse serum, NGF, Ara-C, and conditioned medium as above. Terasaki plates have 60 wells, each of which take about 15 μl of medium. They are very convenient for assays that need relatively few cells. Cultures were used between 2 and 5 days *in vitro*.

Calcium Uptake Assay (29)

Cultured DRG cells were washed four times by flooding Terasaki plates with 10 ml of Hanks' balanced salt solution without calcium or magnesium, buffered to pH 7.4 with 10 mM HEPES (Ca/Mg-free

Hanks'/HEPES). We can measure calcium uptake in physiological concentrations of calcium, but routinely use low concentrations of high specific activity $^{45}Ca^{2+}$ to improve the signal (29). Medium (8 μl) was removed from each well and replaced with 10 μl of Ca/Mg-free Hanks'/HEPES supplemented with 10 μCi/ml $^{45}Ca^{2+}$ and containing test compounds. Stock solutions of capsaicin analogues were made up in dimethyl sulfoxide and diluted 1 to 100 in the Hanks' medium for testing. Dimethyl sulfoxide (final concentration, 1%) was also added to controls. After incubation at room temperature for 8 min, plates were washed six times with 10 ml of Hanks' salt solution containing 10 mM HEPES, pH 7.4 (total Hanks'/HEPES), and dried at 60–70° for about 60 min. A total of 10 μl of 0.1% sodium dodecyl sulfate was pipetted into each well and left at room temperature for at least 15 min. The contents of each well were then transferred to scintillation vials and counted in 2 ml of Beckman CP scintillant. Each experiment included a measurement of background uptake and uptake induced by a maximal (3 μM) concentration of capsaicin.

Photoaffinity Labeling

Cultured DRG cells were washed four times by flooding Terasaki plates with 10 ml of Ca/Mg-free Hanks'/HEPES without phenol red. A total of 8 μl of medium was removed from each well and replaced with 10 μl of the same medium containing photoaffinity label. Cells were incubated in the dark at room temperature for 10 min, and either left in the dark (controls) or irradiated for 2 min with a B-100A Blak-Ray long wavelength (peak 365 nm) UV lamp (Ultra-Violet Products Inc., San Gabriel, CA) held 2 cm from the bottom of the plate. During irradiation there was no measurable increase in temperature underneath the lamp. Cells were then washed eight times with 10 ml of Ca/Mg-free Hanks'/HEPES over a period of about 45 min and uptake of ^{45}Ca was assayed as described above.

Protection Experiments

DRG cells were washed, pretreated with photoaffinity label in the presence of protecting ligand, and irradiated as described above, except that the medium contained 50 μM EGTA to ensure that there was no free calcium (see Discussion). After irradiation, cells were washed twice with 10 ml of Ca/Mg-free Hanks'/HEPES containing 50 μM EGTA, eight times with 10 ml of Ca/Mg-free Hanks'/HEPES containing 10% horse serum and 1 mM EGTA, and finally four times with Ca/Mg-free Hanks'/HEPES. Uptake of ^{45}Ca was then assayed as before. To control for possible desensitization by the protecting agent, cells were either pretreated with photoaffinity label and protector but not irradiated or irradiated in the presence of protector alone before washing and assay.

Measurement of Calcium Concentration

Calcium concentration in buffers was measured using the fluorescent indicator quin2 (32). Calcium standards were prepared by adding $CaCl_2$ to 500 μM EGTA in 0.16 M NaCl, 10 mM HEPES, pH 7.4, assuming a dissociation constant of 398 nM for the Ca-EGTA complex (33). For the assay, 3 μl of quin2 (10 mM in dimethyl sulfoxide) were added to 3 ml of standard or sample and mixed thoroughly. Fluorescence was measured immediately in a Perkin Elmer LS-5 luminescence spectrometer, with the excitation wavelength set at 339 nm and the emission wavelength at 492 nm.

Results

Fig. 1A shows the structures of the three photoaffinity labels. Their activity as reversible ligands is shown in Figs. 2 and 3. These experiments were performed in subdued light so that the azides were not activated. Only one of the analogues (PAL 1) was an agonist in the calcium uptake assay. It was not very potent, reaching only about 40% of the capsaicin maximum at a concentration of 1 mM (Fig. 2). All three compounds inhibited capsaicin-induced uptake. The inhibition reflected a depression

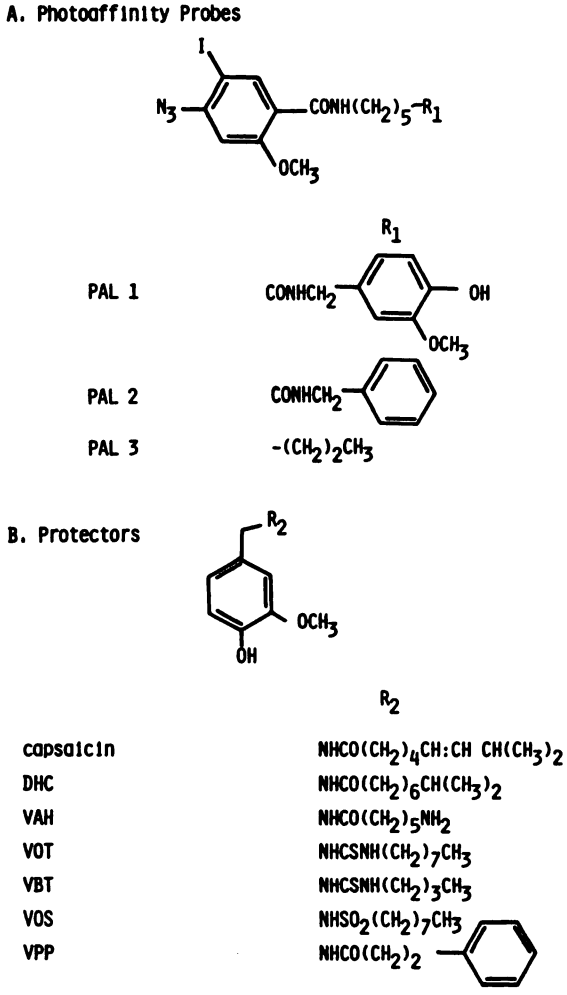

Fig. 1. Structures of capsaicin analogues.

in the maximal response to capsaicin (Fig. 3), suggesting a noncompetitive mechanism. A similar depression in the maximal response to capsaicin was produced by linolenic acid (Fig. 3). Pretreatment of cells with 100 μM photoaffinity labels or with 30 μM linolenic acid (followed by washing as described in Materials and Methods for the photoaffinity labeling experiments) also led to a depression of the maximal response to capsaicin (data not shown).

PAL 1 caused a long-lasting stimulation of calcium uptake if irradiated (to activate the azide) in contact with cells, but not if samples were kept in the dark (Fig. 4). We call this a long-lasting effect because the assay was carried out about 45 min after treatment with PAL 1. This was the time taken to complete the washing procedure. We do not know how long the effect would persist because we could not keep the cells in calcium-free medium for much longer than 45 min; they tended to detach from the plate. Calcium-free medium was essential to avoid problems with desensitization (see below and Discussion). We only saw the long-lasting agonist effect if the azide was activated in contact with the cells. Irradiation of PAL 1 before incubation with cells caused loss of agonist activity. The agonist effects of the nonirradiated compound (see Fig. 1) were clearly reversed by washing the cells (Fig. 4, *DARK*). PAL 1 was more potent as a long-lasting agonist than as a reversible agonist, but the maximal stimulation was only about 50% of the obtainable with capsaicin. The other two aryl azides did

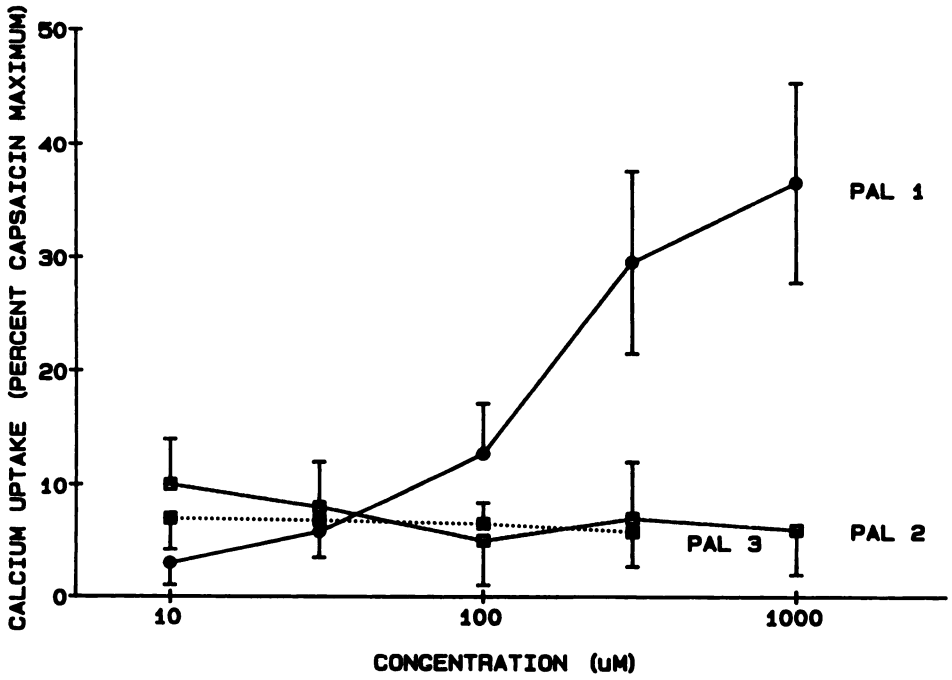

Fig. 2. Agonist activity of capsaicin photoaffinity labels. Calcium uptake assays were performed as described in Materials and Methods. Results are reported as per cent uptake induced by a maximal concentration of capsaicin (3 μM) with cells on the same plate as the test compound. Data are means and standard errors for six independent experiments with PAL 1 and four experiments with PAL 2 and PAL 3. In each experiment measurements were replicated six times.

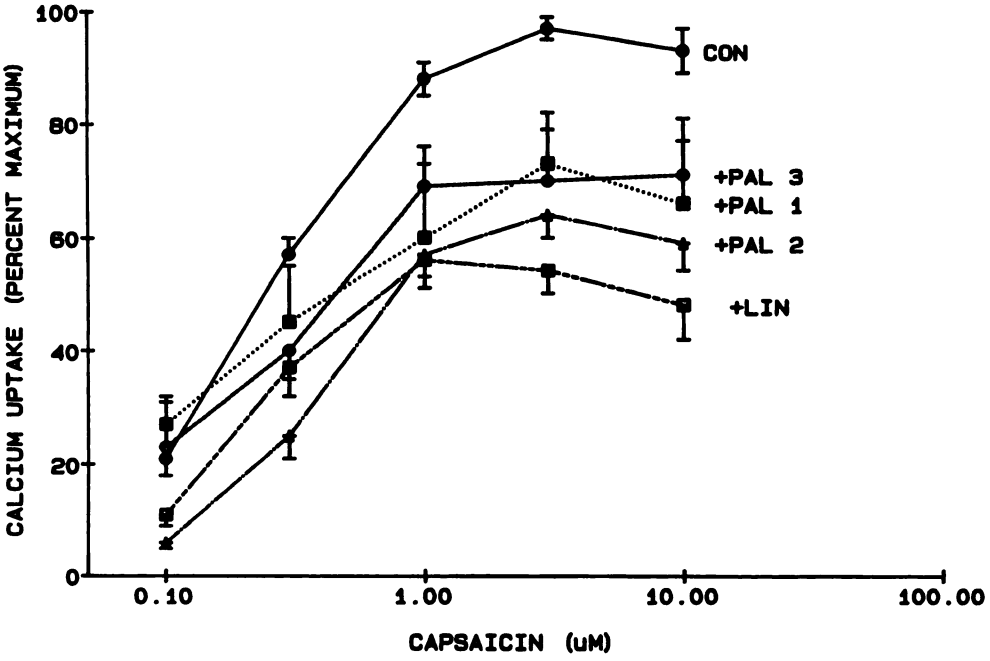

Fig. 3. Effect of photoaffinity labels on the capsaicin dose-response curve. Calcium uptake assays were performed as described in Materials and Methods. For each experiment, a control capsaicin dose-response curve and a curve in the presence of test compound were measured on the same plate of cells. There were six replicates of each measurement. Data are means and standard errors for control dose-response curves (*CON*) (17 experiments) and dose-response curves in the presence of 100 μM PAL 1 (four experiments), 100 μM PAL 2 (four experiments), 100 μM PAL 3 (five experiments), or 30 μM linolenic acid (*LIN*) (four experiments).

not cause long-lasting calcium uptake, even if irradiated. Long-lasting inhibition of capsaicin action was produced by all three compounds. Inhibition by PAL 3 and PAL 2 was enhanced by irradiation, but inhibition by PAL 1 appeared to be independent of irradiation (Fig. 5).

Long-lasting stimulation of calcium uptake with PAL 1 was suppressed if reversible capsaicin agonists were present (as protecting ligands) during irradiation. In these experiments we have used DHC instead of capsaicin as our reference ligand, because [^{3}H]DHC was available to monitor washing procedures (see below). Radioactive capsaicin is not available. In all assays that we have tested, capsaicin and DHC were equipotent. Maximal protection varied from 50 to 100% between preparations of cells. The mean was about 80% when VOT, DHC, or

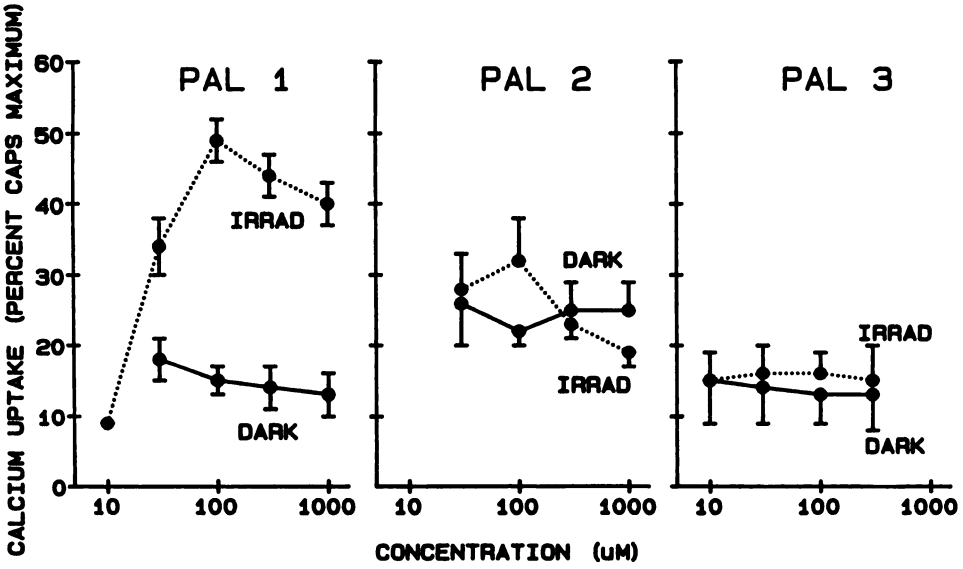

Fig. 4. Long-lasting agonist effects of capsaicin photoaffinity labels. Cells were pretreated with photoaffinity labels, either irradiated or kept in the dark, and washed as described in Materials and Methods. Measurements of background uptake were made after treatment with PAL 1, PAL 2, or PAL 3 by adding Ca/Mg-free Hanks'/HEPES containing 10 μCi/ml $^{45}Ca^{2+}$, incubating for 8 min, and washing as described in Materials and Methods. Data (expressed as per cent capsaicin-induced uptake into untreated cells on the same plate) are means and standard errors for four (PAL 2 and PAL 3) or five (PAL 1) independent experiments. In each experiment measurements were replicated six times.

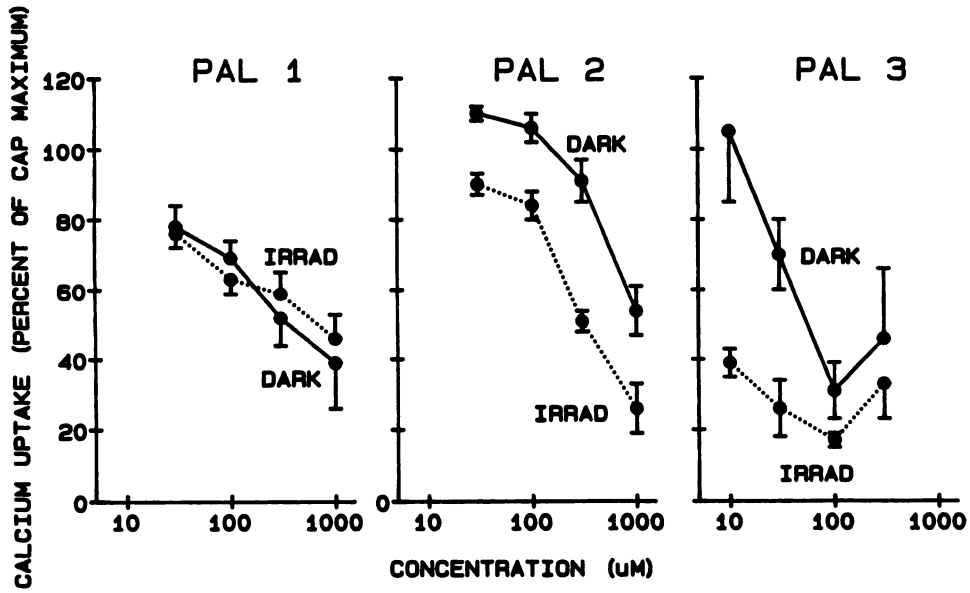

Fig. 5. Long-lasting inhibition of capsaicin action by photoaffinity labels. Cells were treated as for Fig. 4, except that 3 μM capsaicin was added for the calcium uptake assay. Data (expressed as per cent capsaicin-induced uptake into untreated cells on the same plate) are again means and standard errors from four (PAL 2 and PAL 3) or five (PAL 1) experiments.

VOS were used as protectors (Fig. 6; Table 1). We could not use high enough concentrations of VPP or VBT to reach a plateau of protection, because they were insoluble above 300 μM. Assuming that they too would give maximal protection of 80%, there was good agreement between IC_{50} as protectors and EC_{50} as agonists in the calcium uptake assay for a series of five capsaicin analogues. A sixth (VAH) was inactive at concentrations up to 300 μM in both assays.

In the protection experiments, buffers for incubation and washing of the cells contained EGTA to ensure completely calcium-free medium. This was essential for avoiding desensitization by the protecting ligands (see Discussion). Without EGTA, as in the original photoaffinity experiments (Figs. 4 and 5), the calcium concentration, as measured with quin2, was 260 nM, even though the buffer was nominally calcium-free. Addition of 50 μM EGTA reduced the calcium to undetectable levels. In buffers without EGTA, pretreatment with protectors did cause desensitization to capsaicin action in the subsequent calcium assay. In buffers with EGTA added, there was no desensitization, even at concentrations of agonist that gave maximal protection (Table 2). We could detect no difference in washing efficiency between the two procedures. Removal of 100 μM [^{3}H]DHC was (mean ± SE, three experiments) 98.2 ± 0.14% for buffers without EGTA and 98.6 ± 0.32% for buffers with EGTA. Similar results were obtained for concentrations of [^{3}H]DHC down to 0.1 μM. After washing, calcium uptake into cells treated with unactivated PAL 1 (Fig. 4) or with protecting ligands returned to background levels.

Discussion

There are two indirect lines of evidence leading to the suggestion that capsaicin interacts at a selective receptor to produce its effects. The first is the cellular specificity of capsaicin. Both *in vivo* and *in vitro* capsaicin is selective for certain types of sensory neurons. For example, treatment of newborn rats with capsaicin leads to selective loss of C and some Aδ fibers (17,19). *In vivo*, relatively low doses of capsaicin excite C fibers selectively, with few effects on other sensory neurons (23). *In vitro*, capsaicin is selective for a subpopulation of cultured DRG neurons (11, 28, 29). They are the small dark population of

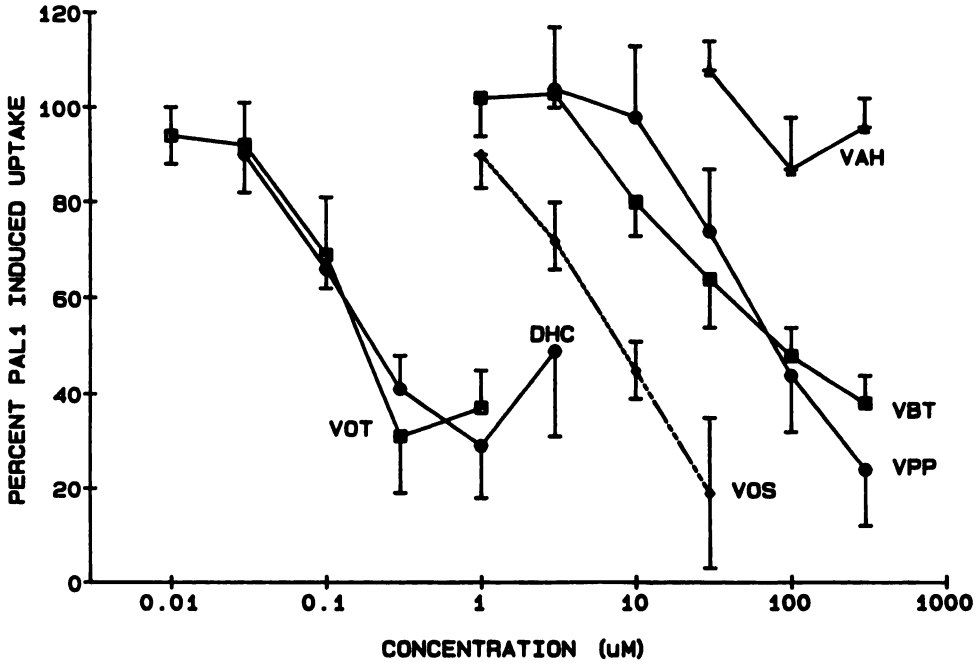

Fig. 6. Protection from PAL 1. Cells were pretreated with 100 μM PAL 1 in the presence of various concentrations of protecting ligands, irradiated, and washed as described in Materials and Methods and calcium uptake was measured in the absence of capsaicin. Data (expressed as per cent uptake induced by irradiation in the presence of PAL 1 alone) are means and standard errors from four independent experiments with six replicates of measurements in each experiment.

TABLE 1

Potency of capsaicin analogues in the calcium uptake assay and as protectors

Calcium uptake and protection assays were performed as described in Materials and Methods. EC_{50} calcium uptake is the concentration of agonist required to produce 50% of the maximal response. All agonists except VAH gave the same maximal response as capsaicin. IC_{50} protection is the concentration of protector required to produce 50% of the maximal obtainable protection. For ligands marked * a plateau was not reached in the protection curve; IC_{50} values were estimated assuming that maximal protection was 80% (see text). Data are means and standard errors from four independent experiments.

Ligand	EC_{50} calcium uptake	IC_{50} protection	Maximal protection
	μM	μM	%
VOT	0.15 ± 0.03	0.12 ± 0.03	77 ± 12
DHC	0.18 ± 0.03	0.15 ± 0.03	81 ± 11
VOS	2.5 ± 0.3	5.6 ± 1.4	84 ± 10
VBT	10 ± 0.9	45 ± 19*	*
VPP	60 ± 4.0	100 ± 59*	*
VAH	>300	>300	*

TABLE 2

Effect of pretreatment with protectors on capsaicin activity

Cells were pretreated with capsaicin analogues and washed as for protection experiments (see Materials and Methods). Uptake of $^{45}Ca^{2+}$ induced by 3 μM capsaicin was measured. Results are expressed as a percentage of uptake into untreated cells on the same plate as those treated with the capsaicin analogue. Data are means and standard errors for the number of experiments in parentheses.

Protector	Concentration	% Capsaicin maximum
	μM	
VOT	1	102 ± 3 (4)
DHC	1	97 ± 4 (4)
VOS	30	86 ± 8 (3)
VBT	300	96 ± 9 (3)
VPP	300	83 ± 16 (3)

DRG neurons and do not stain with antibodies against neurofilaments (29, 30). Cultures of neurons from rat superior cervical ganglia or of nonneuronal cells from sciatic nerve are insensitive to capsaicin (29).

The second line of evidence comes from structure-activity studies. There are fairly strict structural requirements for capsaicin activity in assays of pungency (26), hypothermia, and antinociception (5). These requirements led Szolcsanyi and Jancso-Gabor (26) to propose a model for a putative capsaicin receptor, but direct evidence for such a receptor has been elusive. Measurements of saturable binding with radiolabeled DHC are difficult because the relatively low affinity (based on potency in bioassays, dissociation constants are probably more than 100 nM; Table 1) (see also Ref. 29) and the lipophilic nature of the available radioligands means that nonsaturable binding to membranes is very high. Our approach to avoid at least one of these problems is to use irreversible ligands, in this case photoaffinity labels, for the putative capsaicin receptor.

We have shown that a photoaffinity label based on capsaicin produces long-lasting capsaicin-like calcium uptake in cultured sensory neurons. Irradiation of cells in the presence of the photoaffinity label is essential for the long-lasting stimulatory effect. The structural features of the molecule that are necessary to produce the effect are similar to those required for activity in assays of antinociceptive activity, e.g., a 3-methoxy, 4-hydroxy benzyl ring is needed. The long-lasting stimulation of calcium uptake can be prevented by having reversible capsaicin agonists present during treatment of cells with the photoaffinity label. This activity cannot be explained by a general desensitization to capsaicin. We suggest, therefore, that the reversible agonists are protecting a capsaicin binding site from reaction with the photoaffinity reagent. We further suggest that this binding site corresponds to a capsaicin receptor that mediates at least some of the effects of capsaicin on sensory neurons. We make these suggestions first because of the necessity for certain structural features in the photoaffinity label (see above) and second because the activity of capsaicin analogues as protectors correlates well with their activity as agonists in the calcium uptake assay.

PAL 1 also caused long-lasting inhibition of capsaicin-induced calcium uptake. The overall activity of this reagent is probably a balance between its stimulatory and inhibitory effects. The inhibitory activity was unexpected and we know

very little about the mechanism involved. Inhibition is unlikely to be mediated by the same binding site as the stimulation of calcium uptake, first because there appear to be no strict structural requirements for inhibitory activity, all three photoaffinity reagents and even the unrelated compound linolenic acid were effective inhibitors, secondly because the mechanism of inhibition by the photoaffinity reagents and linolenic acid appears to be noncompetitive, and third because irradiation is not strictly necessary for the inhibitory effects. We used linolenic acid because we suspected that inhibition was caused by the fatty side chains of the photoaffinity reagents. This speculation was supported by the inhibitory activity of PAL 3, which is simply an extension of the PAL 1 side chain. We predicted that other fatty chains unrelated to capsaicin or the photoaffinity reagents would have a similar inhibitory activity. The experiments with linolenic acid confirm this. We also wanted to test linolenic acid as a protector. Our prediction was that because protection is related to agonist activity linolenic acid would be inactive. Unfortunately, we could not wash it out of the cells well enough to allow reversal of the inhibitory effects, so we were unable to do the protection experiments.

Desensitization by protecting ligands was a major problem in our first attempts to show protection from the long-lasting agonist effect of PAL 1. Desensitization does occur with the calcium uptake assay and is totally dependent on the presence of extracellular calcium (31). We found that desensitization could be prevented by including EGTA in the buffers during the exposure to agonist and changing the washing procedure for the protection experiments. The new washing procedure was no more effective at removing [^{3}H]DHC than the original procedure, so it was presumably the inclusion of EGTA that was important. Although the buffers in the original procedure were nominally free of calcium, we measured an actual calcium concentration of 260 nM. The EGTA in the modified procedure removed the calcium and prevented desensitization.

We now hope to identify and characterize molecules from DRG that bind PAL 1 specifically and to examine the possibility that the capsaicin binding site may have a physiological function.

Acknowledgments

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References

1. Jancso, N., A. Jancso-Gabor, and J. Szolcsanyi. Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br. J. Pharmacol.* **31**:138–151 (1967).
2. Carpenter, S. E., and B. Lynn. Vascular and sensory responses of human skin to mild injury after topical treatment with capsaicin. *Br. J. Pharmacol* **73**:755–758 (1981).
3. Konietzny, F., and H. Hensel. The effects of capsaicin on the response characteristic of human polymodal nociceptors. *J. Therm. Biol.* **8**:213–215 (1983).
4. Szolcsanyi, J. Sensory receptors and the antinociceptive effects of capsaicin, in *Tachykinin Antagonists* (R. Hakanson and F. Sundler, eds.). Elsevier, Amsterdam, 45–54 (1985).
5. Hayes, A. G., A. Oxford, M. Renolds, A. H. Shingler, M. Skingle, C. Smith, and M. B. Tyers. The effects of a series of capsaicin analogues on nociception and body temperature in the rat. *Life Sci.* **34**:1241–1248 (1984).
6. Jancso, G., and G. Such. Effects of capsaicin applied perineurally to the vagus nerve on cardiovascular and respiratory functions. *J. Physiol. (Lond.)* **341**:359–370 (1983).
7. Molnar, J., L. Gyorgy, G. Unyi, and J. Kenyeres. Effect of capsaicin on the isolated ileum and auricle of the guinea pig. *Acta Physiol. Acad. Sci. Hung.* **35**:369–374 (1969).
8. Bartho, L., and J. Szolcsanyi. The site of action of capsaicin on the guinea pig isolated ileum. *Naunyn-Schmeideberg's Arch. Pharmacol.* **305**:75–81 (1978).
9. Zernig, G., P. Holzer, and F. Lembeck. A study of the mode and site of action of capsaicin in guinea pig heart and rat uterus. *Naunyn-Schmeideberg's Arch. Pharmacol.* **326**:58–63 (1984).
10. Williams, J. T., and W. Zieglgansberger. The acute effects of capsaicin on rat primary afferents and spinal neurons. *Brain Res.* **252**:125–131 (1982).
11. Baccaglini, P. I., and P. G. Hogan. Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proc. Natl. Acad. Sci. USA* **80**:594–598 (1983).
12. Hayes, A. G., A. B. Hawcock, and R. G. Hill. The depolarizing action of capsaicin on rat isolated sciatic nerve. *Life Sci.* **35**:1561–1568 (1984).
13. Heyman, I., and H. P. Rang. Depolarizing responses to capsaicin in a subpopulation of rat dorsal root ganglion cells. *Neurosci. Lett.* **56**:69–75 (1985).
14. Gamse, R., A. Molnar, and F. Lembeck. Substance P release from spinal cord slices by capsaicin. *Life Sci.* **25**:629–636 (1979).
15. Theriault, E., M. Otsuka, and T. Jessell. Capsaicin-evoked release of substance P from primary sensory neurons. *Brain Res.* **170**:209–213 (1979).
16. Singer, E. A., G. Sperk, and R. Schmid. Capsaicin does not change tissue levels of glutamic acid, its uptake or release in rat spinal cord. *J. Neurochem* **38**:1383–1386 (1982).
17. Jancso, G., E. Kiraly, and A. Jancso-Gabor. Pharmacologically induced selective degeneration of chemosensitive primary neurones. *Nature (Lond.)* **270**:741–743 (1977).
18. Jancso, G., E. Kiraly, F. Joo, G. Such, and A. Nagy. Selective degeneration by capsaicin of a subpopulation of primary sensory neurones in the adult rat. *Neurosci. Lett.***59**:209–214 (1985).
19. Jancso, G., M. Ferencsik, G. Such, E. Kiraly, A. Nagy, and M. Bujdoso. Morphological effects of capsaicin and its analogues in newborn and adult mammals, in *Tachykinin Antagonists* (R. Hakanson and F. Sundler, eds.). Elsevier, Amsterdam, 35–44 (1985).
20. Kenins, P. Responses of single nerve fibres to capsaicin applied to the skin. *Neurosci. Lett.***29**:83–88 (1982).
21. Ueda, N., I. Muramatsu, and M. Fujiwara. Capsaicin and bradykinin-induced substance P-ergic responses in the iris sphincter muscle of the rabbit. *J. Pharmacol. Exp. Ther.* **230**:469–473 (1984).
22. Gamse, R. Capsaicin and nociception in the rat and mouse: possible role of substance P. *Naunyn-Schmiedeberg's Arch. Pharmacol.***320**:205–216 (1982).
23. Fitzgerald, M. Capsaicin and sensory neurones: a review. *Pain* **15**:109–130 (1983).
24. Jones, E. C. S., and F. L. Pyman. The relation between chemical constitution and pungency in acid amides. *J. Chem. Soc.* **127**:2588–2598 (1925).
25. Hegyes, P., and S. Foldeak. Synthesis of homovanillic acid derivatives of capsaicin-like effect. *Acta Phys. Chem.* **20**:115–120 (1974).
26. Szolcsanyi, J., and A. Jancso-Gabor. Sensory effects of capsaicin congenors. I. Relationship between chemical structure and pain-producing potency of pungent agents. *Arzneim. Forsch. Drug Res.* **25**:1877–1881 (1975).
27. Szolcsanyi, J., and A. Jancso-Gabor. Sensory effects of capsaicin congenors. II. Importance of chemical structure and pungency in desensitising activity of capsaicin-type compounds. *Arzneim. Forsch. Drug Res.* **26**:33–37 (1976).
28. Bevan, S. J., I. F. James, H. P. Rang, J. Winter, and J. N. Wood. The mechanism of action of capsaicin—a sensory neurotoxin, in *Neurotoxins and Their Pharmacological Implications: A Biological Council Symposium* (P. Jenner, ed.). Raven Press, New York, 261–277 (1987).
29. Wood, J. N., J. Winter, I. F. James, H. P. Rang, J. Yeats, and S. J. Bevan. Capsaicin induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.*, in press (1988).
30. Winter, J. Characteristics of capsaicin-sensitive neurons in adult dorsal root ganglion cultures. *Neurosci. Lett.* **80**:134–140 (1987).
31. Wood, J. N. Reversible desensitization to capsaicin induced calcium uptake in cultured rat sensory neurones. *Neuroscience* **22**:S247 (1987).
32. Tsien, R. Y., T. Pozzan, and T. J. Rink. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol* **94**:325–334 (1982).
33. Fabiato, A., and F. Fabiato. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol (Paris)* **75**:463–505 (1979).

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