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SOME EFFECTS OF CHEMICAL IRRITANTS ON THE MEMBRANE OF THE GIANT AMOEBA

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1 The effects of chemical irritants on the membrane potential and input resistance of the giant amoeba, *Chaos carolinense*, have been investigated. The membrane potential and input resistance were −111.5 mV and 8.6 MΩ respectively.

2 In the resting state the cell membrane of *Chaos carolinense* was found to be impermeable to Na^+ but permeable to K^+. The distribution of K^+ across the cell membrane conformed to a Donnan equilibrium with the resting membrane potential being the K^+ equilibrium potential.

3 The chemical irritants dibenzoxazepine and its 2-chloro- and 3-chloro-analogues and *o*-chlorobenzylidene malononitrile produced a fall in input resistance but no change in membrane potential. It is suggested that these effects are caused by an increase in K^+ permeability.

4 The potencies of a series of chemical irritants with respect to dibenzoxazepine were measured on the giant amoeba. These potencies did not reflect those found in mammalian preparations.

Introduction

Chemical irritants produce a variety of effects when applied to skin and mucous membranes. These effects include pain, flare, sneezing, coughing, lachrymation and blepharospasm and have resulted in the use of chemical irritants as rubefacients (e.g. capsaicin) and as riot control agents (e.g. *o*-chlorobenzylidene malononitrile (CS)).

It has been shown in both whole animals and isolated mammalian preparations that chemical irritants have a wide spectrum of action. The varied effects of capsaicin have been most fully documented and include actions on cardiovascular, respiratory, gastrointestinal and thermoregulatory systems (for a review, see Virus & Gebhart, 1979). In addition, several studies using mammalian nerve preparations (Pórszász & Jancsó, 1959; Green & Tregear, 1964; Foster & Ramage, 1981) have suggested that chemical irritants exert a selective excitatory effect upon the peripheral nerve endings of some primary afferents.

The mechanism of action of chemical irritants is unclear. It has been suggested on the one hand that substance P and/or 5-hydroxytryptamine (5-HT) is involved in the pharmacological actions of capsaicin (see Virus & Gebhart, 1979) and on the other that these actions are mediated by specific chemoreceptors (Szolcsányi & Jancsó-Gábor, 1975) leading to changes in the electrical properties of cells.

Unicellular organisms, such as the amoeba, also respond to the presence of chemicals in the environment and these responses may result from interaction with specific chemoreceptors in the cell membrane. Such relatively undifferentiated excitable cells may therefore be suitable for the detection and investigation of any fundamental membrane changes directly mediated by chemical irritants. This possibility prompted the present study of the effects of chemical irritants on the transmembrane potential and input resistance of the giant amoeba, *Chaos carolinense*.

A preliminary account of these results has appeared (Foster, Weston & Weston, 1980).

Methods

Experimental organisms

Chaos carolinense (initially obtained from the Culture Centre for Algae & Protozoa, Cambridge) was grown in modified Pringsheim's (MP) solution (Chapman-Andresen, 1962) in a covered glass dish at room temperature (21.5°C). Every week, the cells were sub-cultured into fresh medium and fed with washed *Colpidium striatum* grown in a barley grain infusion. *Chaos carolinense* was deprived of food for 24 h before an experiment.

Electrical measurements

A single microelectrode in conjunction with a high-impedance probe and preamplifier (Mentor N-950) was used for both recording and the passage of

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current. The indifferent electrode was a sintered silver/silver chloride/platinum black disc (Mentor N-9501). The output from the preamplifier was displayed on an oscilloscope (Tektronix) and polygraph (Grass). A stimulator (Grass S48) was used to deliver short (1 ms) rectangular pulses via the Mentor N-950 to the cell. Long (250 or 500 ms) pulses could be delivered to the cell from a second stimulator via a stimulus isolation unit (Grass SIU5) and the Mentor N-950.

D.c. offsets could be cancelled out before impalement by means of the voltage zero suppression facility on the Mentor N-950. The frequency response of the system was optimized by means of a capacitance compensation facility to reduce the attenuation of high frequency components of the input signal.

Electrodes

A 1 mm external diameter self-filling glass microelectrode (Clark Electromedical) was mounted flexibly on a silver/silver chloride wire and positioned by means of a micromanipulator (Leitz) and binocular microscope (Olympus) with zoom magnification up to $\times 80$. When pulled, electrodes had resistances of 20–100 MΩ. The tips were broken off immediately before impalement by touching on a glass surface (Dichter, 1973) thus reducing the resistance to 5–20 MΩ. Electrodes had tip potentials of less than 1 mV. The electrolyte was 3 M KCl solution.

The contribution of the resistance of the electrode, together with that of the fluid surrounding the cell, was balanced out during the course of an impalement by means of the bridge balancing facility on the Mentor N-950 using a 1 ms 1 nA current pulse.

Electrophysiological measurements

All experiments were performed at room temperature (21.5°C) with the amoeba in 10 ml fluid in a glass Petri dish. Cells were allowed to adhere to the base of the dish and were not restrained. An organism was always impaled in its central region and each organism was used only once.

Membrane potential was measured directly by noting the change in potential which occurred on impalement of a cell. Membrane potential changes (value under test conditions − resting value in MP solution) in a hyperpolarizing direction are subsequently shown as negative values; in a depolarizing direction as positive values. The input resistance of the cell membrane plus cytoplasm was determined during impalement by passing a 250 or 500 ms current pulse across the cell membrane. The resulting change in potential was measured when a steady state was attained and input resistance was calculated using Ohm's Law.

In preliminary experiments, a linear current/voltage relationship was found to exist with current strengths up to 2 nA. A 1 nA 500 ms depolarizing pulse was used for the measurement of input resistance except where stated.

Experimental design

Each experiment involved the measurement of resting membrane potential and input resistance in MP solution of each organism in a population of cells. One group of cells was then transferred into MP solution containing the drug under test. At the same time a control series was performed by transferring a second group of cells into MP solution (transfer control) or, where appropriate, into MP solution containing the ethanol vehicle used to aid drug solution (vehicle control). In each case, membrane potential and input resistance were measured at 2, 4 and sometimes 6 min after transfer.

Effect of dibenzoxazepine on membrane potential during the passage of transmembrane current

A series of transmembrane current pulses (500 ms) of increasing amplitude was passed in both a depolarizing and hyperpolarizing direction through the cell in the presence of dibenzoxazepine. The membrane potential during the passage of current was measured. A control experiment of similar design was performed in the absence of dibenzoxazepine.

Assay of chemical irritants and analogues of dibenzoxazepine

The potencies of the 2- and 3-chloro-analogues of dibenzoxazepine and of other chemical irritants relative to that of dibenzoxazepine were assayed in two separate experiments.

A symmetrical (2 + 2)-dose parallel line bioassay design would have been preferred for accuracy. However, the limited aqueous solubility of the analogues of dibenzoxazepine and of *o*-chlorobenzylidene malononitrile prevented the preparation of highly effective concentrations of these drugs so we were constrained to adopt a (2 + 1)-dose assay design.

From a concentration-effect study, two concentrations of dibenzoxazepine (causing approx. 30 and 70% reductions in input resistance; 200 nM and 20 μM) were selected for use as standards in each assay. The 100 fold concentration interval was chosen to guarantee regression of effect on log concentration and the bracketing by the effects of standards of those of test solutions (concentrations causing approx. 50% reductions in input resistance estimated from pilot experiments).

Changes in membrane potential and input resistance were measured after transferring a group of 8 cells into one of the two standard solutions of dibenzoxazepine or into a solution of one of the two analogues. Test and standard solutions all contained 0.25% ethanol and a concurrent vehicle control was performed.

A second assay was conducted in a similar manner to compare one concentration of each of *n*-nonanoylvanillylamine, *o*-chlorobenzylidene malononitrile and capsaicin with two standard solutions of dibenzoxazepine.

Drugs and solutions

MP solution (Chapman-Andresen, 1962), pH 6.6, had the following composition (mM): Ca^{2+} 0.85, Mg^{2+} 0.08, Na^{+} 0.22, K^{+} 0.35, Fe^{2+} 0.007, NO_3^- 1.7, SO_4^{2-} 0.09, $HPO_4^{2-}/H_2PO_4^-$ 0.11, Cl^- 0.35. NaCl or KCl were added to MP solution in order to raise the concentration to 20 mM.

The 3 M KCl electrolyte solution was prepared from Ultrar grade KCl (Hopkin & Williams) and was filtered (Millipore) before use.

Drugs used were capsaicin (Sigma Chemicals) and dibenzoxazepine, *n*-nonanoylvanillylamine, *o*-chlorobenzylidene malononitrile, 2-chloro-dibenzoxazepine and 3-chloro-dibenzoxazepine (all kindly supplied by the Chemical Defence Establishment, Porton Down). A stock solution of each drug was prepared in 100% ethanol and dilutions were made in MP solution as required.

Statistical methods

Membrane potential data were normally distributed and standard parametric methods were used. Input resistance data, which were not normally distributed, were analysed parametrically after logarithmic transformation of the ratio of resistance after treatment to initial resistance. This transformation produced normality of distribution and reduced coefficient of variation.

Results

Electrophysiological measurements

A record of a typical impalement of *Chaos carolinense* in MP solution is shown in Figure 1. On penetration with the microelectrode, an immediate negative potential was observed. This was often followed by a rapid reversal of potential after which there was a slower return to the initial negative value. The resting membrane potential of *Chaos carolinense* in MP solution was -111.5 ± 0.52 mV (mean ± s.e., $n = 256$) and the input resistance was 8.6 (8.4, 8.9) MΩ (median with limits of s.e.).

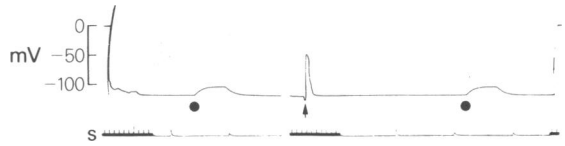

Figure 1 Record of a typical impalement of *Chaos carolinense* in MP solution showing two sections of a continuous recording. Dots indicate passage of 500 ms 1 nA depolarizing pulse for the measurement of input resistance. At arrow, spontaneous electrical event. Note the use of two chart speeds. The small deflections which occur every s on the timer trace are grouped into 5 s intervals (medium deflection) and into 1 min intervals (large deflection).

In some cells large slow depolarizations, lasting approximately 5 s, were occasionally seen (see Figure 1).

In no experiment was there any significant difference in the values for either resting membrane potential or resting input resistance between test and control groups.

Effect of dibenzoxazepine

Membrane potential and input resistance were measured in the presence of dibenzoxazepine at 2, 4 and sometimes 6 min after transfer. The values obtained were found to be independent of the time of measurement and all values were used in the analysis of the results.

In the presence of dibenzoxazepine 50 μM plus ethanol 0.05% the value for the membrane potential was no different from that after transfer into the vehicle control (ethanol 0.05%) but the input resistance ratio (1 nA, 250 ms pulse) was significantly lower (Figure 2).

In a second experiment, the effects of a range of concentrations of dibenzoxazepine (10 pM to 1 mM in ethanol up to 1%) were compared with those of a range of appropriate vehicle controls (ethanol up to 1%). Only dibenzoxazepine 10 μM produced a change in membrane potential (-6 ± 1.4 mV) significantly different from that in the appropriate vehicle control (0 ± 2.4 mV) ($P = 0.04$). Dibenzoxazepine 10 μM and 1 mM produced a significant fall in input resistance ($P < 0.001$) when compared each to its own vehicle control but dibenzoxazepine 10 pM to 100 nM did not. The concentration-effect curve of dibenzoxazepine is shown in Figure 2.

Effect of dibenzoxazepine on membrane potential during the passage of transmembrane current

The membrane potential of *Chaos carolinense* was

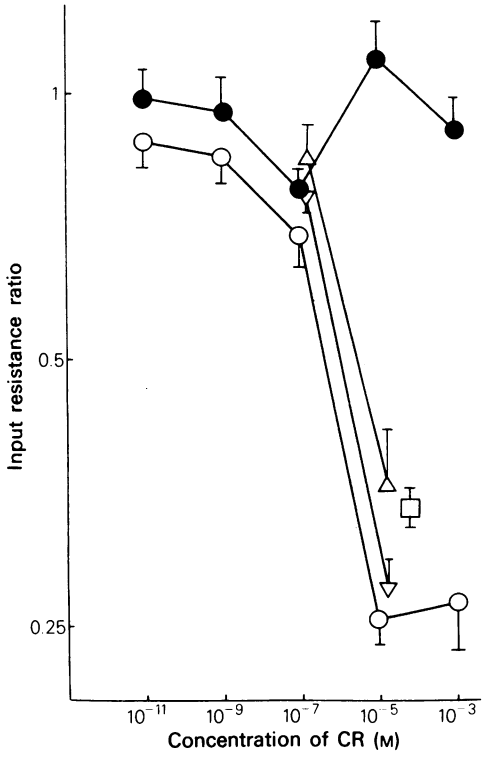

Figure 2 Concentration-dependence of the effect of dibenzoxazepine on input resistance in *Chaos carolinense*. Each point shows the ratio of input resistance after treatment with dibenzoxazepine to that before on a $\log_{10}$ scale (mean values are shown, vertical lines indicate s.e., n not less than 6). Ratios obtained for dibenzoxazepine during time course studies (□), and when used as standard in assays of dibenzoxazepine analogues (▽) and other chemical irritants (△), and in the concentration-effect experiment (○); (●) shows the vehicle control ratios obtained during the concentration-effect experiment.

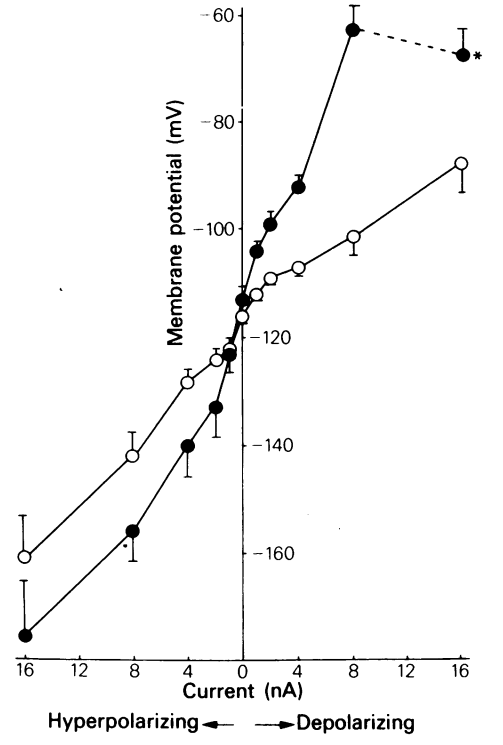

Figure 3 Effect of dibenzoxazepine on the current-voltage relationship in *Chaos carolinense*. (○) Membrane potential (mV, mean values are shown, vertical lines indicate s.e.) in the presence of dibenzoxazepine 10 μM. (●) = Vehicle control. Each point is the mean of 8 observations except *where $n = 4$.

measured during the passage of depolarizing and hyperpolarizing currents across the cell membrane in MP solution containing dibenzoxazepine 10 μM in 0.01% ethanol. A control experiment was performed on cells bathed in MP solution containing the ethanol vehicle only. The current-voltage relationship in the presence and absence of dibenzoxazepine is shown in Figure 3.

Whilst a linear current-voltage relationship occurred in both test and control groups at low current strengths, rectification occurred in both groups at high current strengths. The slope of the linear portion of the curve (= input resistance) in the presence of dibenzoxazepine was less than that in its absence (4.4 and 9.9 MΩ respectively).

Effect of 20 mM NaCl

After transfer into MP solution containing 20 mM NaCl, the value for membrane potential was not significantly different from that in the transfer control. A significant increase in input resistance occurred in the presence of 20 mM NaCl (Table 1).

Effect of 20 mM NaCl plus dibenzoxazepine

Initially, cells were immersed in MP solution containing 20 mM NaCl for 5 min after which resting membrane potential and input resistance were measured. After transfer into MP solution containing 20 mM NaCl plus dibenzoxazepine 50 μM in 0.05% ethanol, the value for membrane potential was not significantly different from that after transfer into the vehicle control (20 mM NaCl plus ethanol 0.05%) but the input resistance ratio was significantly lower (Table 1).

Table 1 Effect of chemical irritants and ionic changes on membrane potential (a negative entry indicates a hyperpolarization) and input resistance

	Change in membrane potential (mV) (mean ± s.e.)	*Input resistance ratio* (median with limits of s.e.)
NaCl 20 mM	−0.8 ± 1.8	1.83 (1.70, 1.96)
Control	−2.3 ± 0.8	1.15 (1.09, 1.23)
P	0.5	< 0.001
NaCl 20 mM + dibenzoxazepine 50 μM	−4.6 ± 2.3	0.35 (0.33, 0.37)
Control	−0.8 ± 2.9	1.28 (1.16, 1.41)
P	0.3	< 0.001
KCl 20 mM	96.5 ± 2.1	0.19 (0.16, 0.22)
Control	−4.3 ± 3.2	1.26 (1.15, 1.39)
P	< 0.001	< 0.001
2-Chloro-dibenzoxazepine 5 μM	−5.7 ± 1.9	0.54 (0.50, 0.58)
Control	1.4 ± 2.5	1.0 (0.96, 1.14)
P	0.03	< 0.001
3-Chloro-dibenzoxazepine 2 μM	4.9 ± 1.8	0.43 (0.39, 0.48)
Control	2.4 ± 2.9	1.0 (0.92, 1.11)
P	0.5	< 0.001
n-Nonanoylvanillylamine 200 μM	1.6 ± 1.9	0.87 (0.82, 0.92)
Control	4.4 ± 2.0	1.1 (0.98, 1.29)
P	0.7	0.1
o-Chlorobenzylidene malononitrile 100 μM	2.3 ± 1.4	0.37 (0.33, 0.42)
Control	4.4 ± 2.0	1.1 (0.98, 1.29)
P	0.6	< 0.001
Capsaicin 200 μM	1.4 ± 2.8	0.78 (0.68, 0.89)
Control	4.4 ± 2.0	1.1 (0.98, 1.29)
P	0.6	0.07

n = not less than 7.

Effect of 20 mM KCl

After transfer into MP solution containing 20 mM KCl, the values for both membrane potential and input resistance were significantly lower than in the transfer control (Table 1).

Effect of analogues of dibenzoxazepine

The effects of 2- and 3-chloro-dibenzoxazepine on membrane potential and input resistance were compared with those of two concentrations of dibenzoxazepine selected from the concentration-effect study.

The values for membrane potential after transfer into dibenzoxazepine (200 nM and 20 μM) and 3-chloro-dibenzoxazepine (2 μM) all in 0.25% ethanol were not significantly different from those after transfer into the vehicle control (0.25% ethanol). A significant hyperpolarization occurred in the presence of 2-chloro-dibenzoxazepine (5 μM). The input resistance ratios after transfer into dibenzoxazepine 20 μM and into 2- and 3-chloro-dibenzoxazepine were significantly lower than for the vehicle control but that for dibenzoxazepine 200 nM was not. The results are shown in Figure 2 and Table 1.

Effect of n*-nonanoylvanillylamine,* o*-chloro-benzylidene malononitrile and capsaicin*

The effects of *n*-nonanoylvanillylamine, *o*-chlorobenzylidene malononitrile and capsaicin on membrane potential and input resistance were similarly compared with those of two concentrations of dibenzoxazepine.

The values for membrane potential after transfer into dibenzoxazepine (200 nM and 20 μM), *n*-nonanoylvanillylamine (200 μM), *o*-chlorobenzylidene malononitrile (100 μM) and capsaicin (200 μM) all in 0.25% ethanol were not significantly different from those after transfer into the vehicle control (0.25% ethanol). The input resistance ratios after transfer into dibenzoxazepine 20 μM and into *o*-chlorobenzylidene malononitrile were significantly lower than into the vehicle control but those for dibenzoxazepine 200 nM, *n*-nonanoylvanillylamine and capsaicin were not. The results are shown in Figure 2 and Table 1.

Relative potency of irritants

From the last two experiments, it was possible to calculate the potencies of the irritants tested on the giant amoeba relative to dibenzoxazepine. The results are shown in Table 2.

Discussion

The values for both resting membrane potential and input resistance in the present study are higher than those cited in earlier literature (Riddle, 1962; Tasaki & Kamiya, 1964; Bruce & Marshall, 1965). However, the culture conditions for the organisms in these investigations were different from those used in the present study and it is possible that discrepancies arise from differences in the characteristics of the recording system and electrodes used. A difference in the value for membrane potential probably resulting from a difference in the technique for impaling cells has been demonstrated in *Amoeba proteus* (Joseffson, 1966; Joseffson, Holmer & Hansson, 1975).

The slow depolarizations seen during impalement in the present study were first described in *Chaos carolinense* by Tasaki & Kamiya (1964). Since such 'spike potentials' could also be observed with extracellular electrodes, these workers suggested they were associated with transmembrane currents. In the present investigation, the depolarizations could not be associated with any visible change in the state of the cell, e.g. movement.

Few of our treatments caused changes in membrane potential; only KCl 20 mM had a dramatic effect. Although significant changes were seen in the presence of 2-chloro-dibenzoxazepine and dibenzoxazepine 10 μM they were small (a few mV).

Changes in input resistance occurred with several of the experimental treatments. The low value seen in the presence of dibenzoxazepine led us to infer that the irritant might affect one or more ionic channels in the cell membrane. Since much of the information available on ionic permeabilities in the giant amoeba derives from work which reports much lower values for resting membrane potential than ours (Riddle, 1962; Bruce & Marshall, 1965) additional experiments were conducted in the presence of high concentrations of NaCl or KCl to verify the information available.

The results of the experiments with high $[NaCl]_o$ in the present investigation suggest that, in the resting state, *Chaos carolinense* is impermeable to Na^+ and Cl^-, confirming the views of other workers (Riddle, 1962; Bruce & Marshall, 1965). The fall in membrane potential in the presence of high $[KCl]_o$ demonstrates that the cell membrane is permeable to K^+. Such a fall could not be attributed to the concommitant increase in Cl^- concentration or osmotic pressure since this would have been revealed in the experiments with high $[NaCl]_o$. Using published values (30–35 mM) for the internal concentration of K^+ (Riddle, 1962; Chapman-Andresen & Dick, 1962; Bruce & Marshall, 1965), the values for the K^+ equilibrium potential in MP solution and in 20 mM KCl (−113 to −117 and −12 mV respectively) predicted from the Nernst equation are similar to those found in the present study (−111.5 and −19 mV respectively). It thus appears that the cell membrane of *Chaos carolinense* is freely permeable to K^+ and that the distribution of K^+ across the cell membrane conforms to a Donnan equilibrium.

An incidental finding in this series of experiments was the increase in input resistance in the presence of NaCl 20 mM. It is possible that this was due to an osmotic effect since Brandt & Freeman (1967) found an increase in input resistance in the presence of 20 mM sucrose. However, further work would be necessary to clarify this.

Since the resting membrane potential in *Chaos carolinense* is the K^+ equilibrium potential and since dibenzoxazepine causes a fall in input resistance unaccompanied by a change in membrane potential, we suggest that dibenzoxazepine (and the other active chemical irritants) produces its effects in the giant amoeba by opening K^+ channels in the cell membrane. It is unlikely that such a selective increase in K^+ permeability would be associated with a direct excitatory mode of action of chemical irritants in mammalian sensory neurones. However, such a permeability change could be associated with any indirect mechanism of action involving the release of mediators.

Table 2 Relative potency of irritants expressed as (a) $\log_{10}$ concentration ratio (± 95% confidence interval) and (b) concentration ratio (with 95% confidence limits)

	(a)	*(b)*
Dibenzoxazepine	0	1.0
3-Chloro-dibenzoxazepine	0.10 ± 0.35	1.26 (0.56, 2.8)
o-Chlorobenzylidene malononitrile	−0.77 ± 0.85	0.17 (0.02, 1.2)
2-Chloro-dibenzoxazepine	−0.78 ± 0.30	0.17 (0.08, 0.33)
Capsaicin	< −1.95	< 0.01
n-Nonanoylvanillylamine	< −2.4	< 0.004

The potencies of the irritants found in decreasing input resistance in the amoeba do not reflect those found in mammalian preparations. In the latter, 2- and 3-chloro-dibenzoxazepine are less potent than dibenzoxazepine whereas *n*-nonanoylvanillylamine and capsaicin have potencies comparable with that of dibenzoxazepine (Foster & Ramage, 1981; N. Creasey, personal communication), although it must be admitted that the published information on the relative potencies in the mammal is both sparse and imprecise. This lack of correlation of potency leads us either to deny the suitability of *Chaos carolinense* as a model of the excitation produced by chemical irritants in sensory neurones or to suggest that *n*-nonanoylvanillylamine and capsaicin differ fundamentally from the other irritants in their site or mechanism of action.

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