

RESINIFERATOXIN, A PHORBOL-RELATED DITERPENE, ACTS AS AN ULTRAPOTENT ANALOG OF CAPSAICIN, THE IRRITANT CONSTITUENT IN RED PEPPER

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Abstract—Resiniferatoxin is an extremely irritant diterpene present in the latex of several members of the genus *Euphorbia*. Its mechanism of action has been shown to be clearly distinct from that of the structurally related phorbol esters. Since resiniferatoxin possesses a 4-hydroxy-3-methoxyphenyl substituent, a key feature of capsaicin, the major pungent ingredient of plants of the genus *Capsicum*, we examined the ability of resiniferatoxin to induce typical capsaicin responses. We report here that treatment of rats with resiniferatoxin, like treatment with capsaicin, caused hypothermia, neurogenic inflammation, and pain. These responses were followed by loss of thermoregulation, by desensitization to neurogenic inflammation, and by chemical and thermal analgesia, with cross-tolerance between resiniferatoxin and capsaicin. Resiniferatoxin was 3–4 orders of magnitude more potent than capsaicin for the effects on thermoregulation and neurogenic inflammation. Resiniferatoxin was only comparable in potency to capsaicin, however, in the assay for induction of acute pain, and the desensitization to acute pain appeared to require less resiniferatoxin than did desensitization for the other responses.

We conclude that resiniferatoxin acts as an ultrapotent capsaicin analog and hypothesize that it may distinguish between subclasses of capsaicin response.

Resiniferatoxin (RTX) is a naturally occurring diterpene, structurally related to the phorbol esters, which was identified in the latex of three species of *Euphorbia* (*E. resinifera*, *E. poissonii*, and *E. unispina*).^{11,21} RTX was isolated on the basis of extraordinary activity in the mouse ear reddening assay,¹¹ in which it exhibited three orders of magnitude greater potency than the most potent of the typical phorbol esters, phorbol 12-myristate 13-acetate (PMA, also abbreviated TPA).^{1,10} Characterization of RTX strongly argued that its primary target was distinct from that of the phorbol esters. Unlike the persistent inflammation induced by the phorbol esters, the ear reddening induced by RTX was transient.^{1,12,22} RTX was not tumor promoting;²⁷ RTX did not induce typical phorbol ester responses in cultured cell systems;^{4,27} and RTX did not efficiently compete for phorbol ester binding to protein kinase C.⁵

A free 20-hydroxyl group is critical for phorbol ester activity.¹⁰ RTX, in contrast, is esterified at this position with 4-hydroxy-3-methoxyphenylacetate, and this substituent has been shown to be essential for its unusual irritant activity.^{1,22}

Strikingly, a 4-hydroxyl-3-methoxyphenyl substituent is also a critical feature of capsaicin, the major pungent constituent of red pepper and other plants of the genus *Capsicum*.^{15,24} (Fig. 1). Structure–activity analysis of capsaicin congeners, moreover, indicates that an ester linkage such as is found in RTX is adequately tolerated in place of the amide linkage present in the opposite orientation in capsaicin.

Capsaicin causes diverse physiological effects, of which pain, neurogenic edema, and hypothermia are prominent examples.^{2,15,24} Acute response is followed by long-lasting desensitization. The postulated cellular mechanism is by transient activation followed by desensitization and, under some conditions, degeneration of C-fiber sensory afferent neurons.^{2,15,20} These neurons contain substance P and associated neuropeptides. The biochemical mechanism for this effect of capsaicin on C-fibers is not known.

Since preliminary studies from this laboratory confirmed the possible homology of RTX and capsaicin—i.e. both compounds produced a dramatic fall in body temperature in mice followed by cross-tolerance³—we have now compared the potencies and *in vivo* activities of RTX and capsaicin in detail.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats (females, 250–300 g) were purchased from the Zivic-Miller Laboratories, (Zelienople, PA). Animals were allowed access to food and water *ad libitum* through the course of the experiments. The numbers of animals used in different experimental groups are indicated in the table and figure legends. RTX (MW 628) was obtained from Chemicals for Cancer Research, Inc. (Chanhassen, MN) or from Chemsyn Science Laboratories (Lenexa, KS) and capsaicin (MW 305) from Polysciences (Warrington, PA). The compounds were administered in 10% ethanol/10% Tween-80/80% physiological saline unless otherwise indicated.

Acute pain-inducing potency of the compounds was assessed as described by Jancso *et al.*¹³ Briefly, solutions at 10-fold increasing concentrations of compound in physiological saline were dropped into the eyes of rats and the number of protective movements (eye-wipings with the foreleg) was determined. To minimize the irritant being

Abbreviation: RTX, resiniferatoxin.

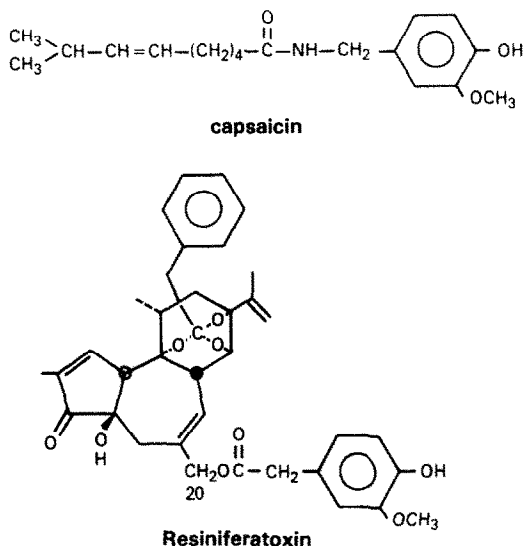


Fig. 1. Comparison of the structures of capsaicin and resiniferatoxin.

rinsed out by lacrimation, the instillation was repeated once. The solvent by itself induced no wipings. The concentrations inducing an equal reaction of 10 wipings (moderate pain-producing potency) were calculated from the dose-response curves.²⁴ Except for these experiments, both RTX and capsaicin were applied under light ether anesthesia to avoid unnecessary pain.

Changes in vascular permeability to serum albumin were analysed using the Evan's Blue technique.¹⁸ Rats were anesthetized with pentobarbitone (40 mg/kg), the right jugular vein was exposed, and 20 mg/kg of Evan's Blue (1% Evan's Blue solution in physiological saline containing 100 IU/ml heparin) was injected.¹⁸ At the indicated times after injection, the animals were killed and the tissues excised. The excised tissues were blotted with filter paper to remove excess fluid, immediately weighed, and then placed in 4.0 ml formamide for 24 h at 50°C to extract the Evan's Blue. Evan's Blue was quantitated by determining absorption at 620 nm in a Shimadzu spectrophotometer.

The extent of edema was measured by treating the hind paw topically with the irritant or solvent. At the indicated time the animal was killed, 1-cm diameter skin punches were removed and quickly weighed, and after drying for 24 h at 50°C the skin punches were reweighed. The difference in the water content between irritant- and solvent-treated animals represents the extent of edema.⁷

To obtain direct evidence for the neurogenic origin of the observed inflammation, denervated rats were purchased from Zivic-Miller Laboratories. Experiments were carried out 3 days after the left saphenous nerve had been cut in the upper thigh.¹⁴ Irritants were applied on both hind paws; the right intact paw served as a positive control.

Body temperature was determined as described by Szikszay *et al.*²³ Rats were habituated prior to treatment to the room temperature (20°C) and to the small-animal temperature probe (Cole-Parmer, Chicago, IL) which was introduced rectally to a depth of 5 cm.

Desensitization against acute chemical pain was examined using the eye-wiping test. RTX was injected s.c. and 4 h later capsaicin was instilled into the eye. Antinociceptive effect against chronic pain was tested by the method of Dubuisson and Dennis.⁶ Five percent formalin solution was injected under the plantar surface of the right forepaw 4 h after topical RTX or capsaicin administration. Behavior of animals was scored during an observation period of 60 min. Heat nociception was examined in the tail-immersion test at

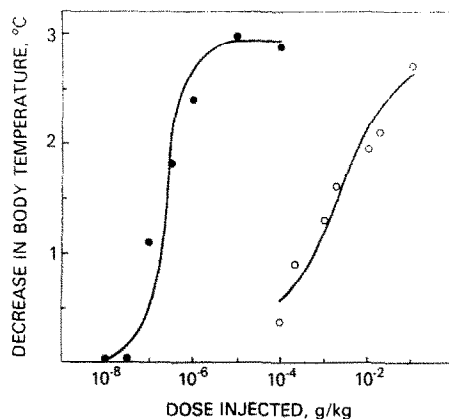


Fig. 2. Induction of hypothermia by RTX or capsaicin. The fall in rectal temperature was measured 1 h after the s.c. injection of RTX (●) or capsaicin (○) at the indicated doses. Points represent the mean for 10 animals in two experiments.

50°C.⁸ Desensitization against the inflammatory response was determined by measuring extravasated Evan's Blue after a hind paw had been painted with xylene, an effective inducer of neurogenic inflammation.¹⁴ In other experiments, dextran was injected under the hind paw skin (25 µg in a volume of 50 µl); dextran releases vasoactive substances without causing neurogenic inflammation.¹⁴

RESULTS

Capsaicin has profound effects on thermo-regulation in mammals.^{16,17,25,26} RTX, like capsaicin, caused a 2–3°C drop in rectal temperature 1 h after treatment of rats maintained at 20°C (Fig. 2). The ED₅₀ for RTX was 3×10^{-7} g/kg, compared to an ED₅₀ for capsaicin of 2×10^{-3} g/kg.

Repeated treatment with capsaicin causes complete desensitization to its hypothermic effects.^{15,26} Similarly, treatment with RTX at a dose of 1×10^{-4} g/kg caused an abrupt decrease in body temperature which largely returned to control levels after 3 h (Fig. 3A). A second treatment with RTX caused only a limited response and further treatments had no effect. The desensitized animals showed cross-desensitization to capsaicin (10^{-2} g/kg), whereas control animals showed marked hypothermia to capsaicin as expected.

Capsaicin-desensitized rats display an inability to adapt to heat stress.^{15,25} If subjected to elevated ambient temperature (38°C), RTX-desensitized rats showed a steady rise in body temperature, ultimately leading to collapse, in contrast to control animals (Fig. 3B).

Desensitization with capsaicin can be achieved by means of either a single large dose or repeated incremental doses.^{2,15} The former procedure is more convenient for quantitation and comparison. We therefore determined the dose dependency for desensitization to RTX-induced hypothermia following pretreatment with a single, s.c. injection of RTX (Fig. 4). The dose-response curve was extremely steep and yielded an ED₅₀ of 5×10^{-6} g/kg. This value is

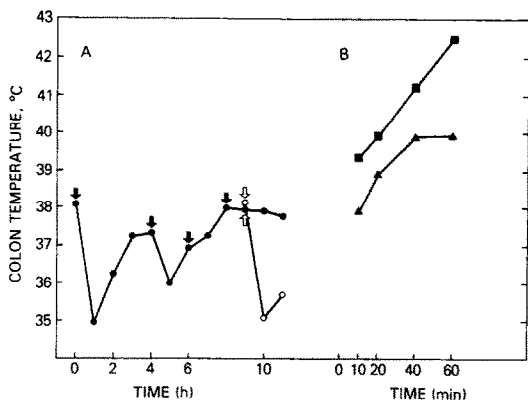


Fig. 3. Body temperature as a function of time after administration of RTX. A. Animals were injected with 100 $\mu\text{g/kg}$ of RTX at the times indicated by the solid arrows (\bullet). Cross-tolerance was examined by injection of 10 mg/kg capsaicin (open arrows). Vehicle controls were injected with the same dose of capsaicin (\circ). Values are the mean for 6–8 animals in two experiments. B. Vehicle controls (\blacktriangle) or animals desensitized by multiple subcutaneous injections with a cumulative dose of 400 $\mu\text{g/kg}$ RTX (\blacksquare) were exposed to high ambient temperature (38°C). Hypothermia resulted in collapse of the rats. Values are the mean for 8 animals in two experiments.

approximately an order of magnitude greater than the ED_{50} for the direct hypothermic effect, but similar to that which gives a maximal response.

Induction of neurogenic inflammation is a second prominent feature of the capsaicin response.^{14,17,25} We quantitated inflammation by measuring extravasation of Evan's Blue dye. Like capsaicin, RTX applied topically to the hind paw of rats caused dramatic extravasation. The ED_{50} for topical administration of RTX was 3×10^{-5} g/paw, compared to an ED_{50} for capsaicin of 3×10^{-2} g/paw (Fig. 5). The kinetics of edema formation were determined from the water content of the hind paw skin as a function of time. No edema formation was observed up to 30 min; the water content reached its peak at 90 min; and the edema disappeared by 4 h (data not shown). The solvent by itself did not have any effect.

Since the inflammation caused by capsaicin is neurogenic, we compared the inflammatory response to RTX in intact paws and in paws which had been denervated by transection of the saphenous nerve in the thigh of the rat. Denervation totally blocked the inflammatory response to RTX, measured either by Evan's Blue extravasation or by increase in water content (Table 1).

Although we quantitated Evan's Blue extravasation only following topical treatment of the paw with RTX, we visually examined systemic extravasation after intravenous injection of RTX (0.1 $\mu\text{g/kg}$, data not shown). RTX induced a similar pattern of extravasation to that reported for capsaicin:¹⁹ extravasation in skin, lung, ovary, and most other tissues, but characteristically not in stomach and intestine.

Capsaicin treatment is followed by desensitization to neurogenic inflammation.^{2,15,17,25} Injection of RTX

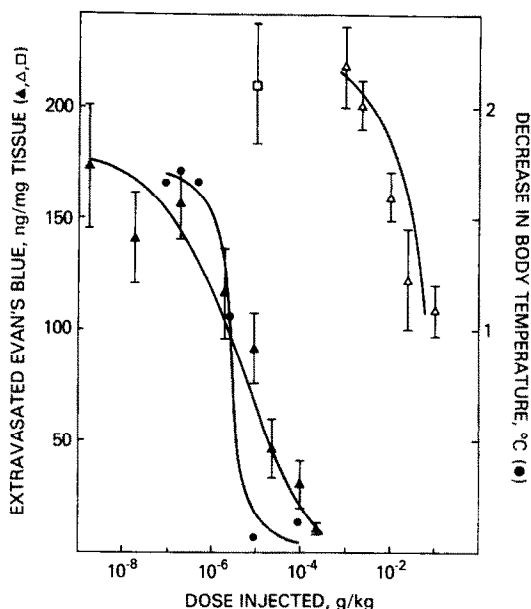


Fig. 4. Dose dependence of desensitization to hypothermic action and Evan's Blue extravasation. Extravasation was induced by xylene (50 $\mu\text{l/paw}$) 4 h after RTX (\blacktriangle) or capsaicin (\triangle) was injected s.c. at the indicated doses. Values are the mean \pm S.D. for 8–12 animals in three experiments. Extravasation was measured 5 min after xylene application. \square Represents xylene-induced Evan's Blue extravasation in vehicle controls. Hypothermia was induced by injection of 100 $\mu\text{g/kg}$ RTX, a dose providing a maximal response, 12 h after RTX pretreatment (\bullet) at the indicated doses. The time interval was chosen to avoid any interference between hypothermia produced by the pretreatment and the test dosage. Values are the mean for 8–10 animals in two experiments.

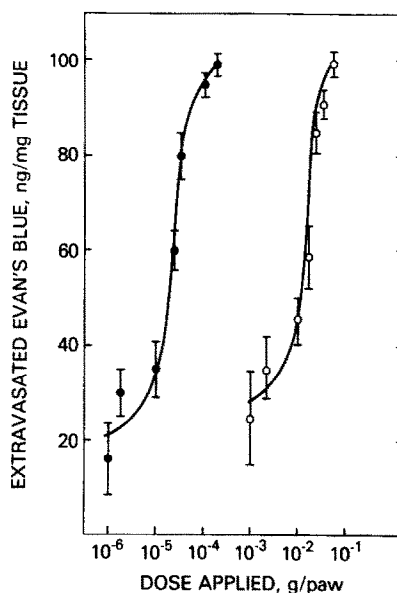


Fig. 5. Dose dependence of Evan's Blue extravasation induced by topical RTX or capsaicin. RTX (\bullet) or capsaicin (\circ) at the indicated doses was painted on the skin of the hind paw in 50 μl acetone. Extravasation was measured 30 min after application. Points are the mean \pm S.D. for 10 animals in two experiments.

Table 1. Effect of denervation on resiniferatoxin-induced edema

Treatment	
Extravasated Evan's Blue (ng/mg tissue)	
Solvent only	11.3 ± 2.5
80 µg RTX on denervated paw	8.9 ± 1.4
80 µg RTX on intact paw	84.2 ± 11.4
Water content of skin (mg/punch)	
Solvent only	40 ± 10
50 µg RTX on denervated paw	39 ± 14
50 µg RTX on intact paw	72 ± 21

The left saphenous nerve was cut 3 days before the experiment. RTX was applied topically in 50 µl acetone on both hind paws; the intact paw served as a positive control. Values are the mean ± S.D. for 10–14 animals in two experiments. Extravasation of Evan's Blue and water content of skin were measured 30 and 90 min after RTX application, respectively.

subcutaneously likewise prevented Evan's Blue extravasation upon challenge 4 h later with either RTX or capsaicin. RTX pretreatment similarly blocked response to xylene, another potent neurogenic irritant,¹⁴ but had little effect on the response to dextran, a potent irritant with a non-neurogenic mechanism (data not shown).

The desensitization induced by s.c. injection of RTX was rapidly expressed and of long duration (data not shown). Inhibition of neurogenic inflammation was greater than 70% by 1 h and achieved a plateau level by 4 h which was maintained over the next 4 days. Responsiveness only partially returned by 7 days after treatment.

The ED₅₀ for inhibition of xylene-induced Evan's

Blue extravasation by s.c. injection of RTX was 5 × 10⁻⁶ g/kg (Fig. 4). That for capsaicin was 10⁻¹ g/kg. In the case of capsaicin, the maximum tolerated s.c. dose afforded only partial desensitization upon a single administration, whereas complete desensitization was attainable with RTX.

Capsaicin potently stimulates chemogenic pain receptors,^{15,17} and the irritancy to the eye has been utilized most extensively to quantitate capsaicin structure-activity relations.^{13,24} As in the other assays, RTX proved to be more potent than capsaicin (Fig. 6A). However, the difference in potency was much less than the 10³–10⁴-fold difference observed for the hypothermic and inflammatory effects. In addition, a latency period of 5–10 s was observed for RTX treatment compared to an immediate response upon capsaicin instillation, and the duration of the response was longer. Extension of the dose-response curves for the eye-wiping response to higher levels was not carried out so as to avoid undue pain.

Injection of RTX s.c. caused desensitization of the eye-wiping response to capsaicin (Fig. 6B). The ED₅₀ for RTX was 1 × 10⁻⁷ g/kg, a dose significantly less than that for desensitization to the hypothermic and inflammatory effects. This difference was confirmed in animals treated with RTX at 1 × 10⁻⁶ g/kg and assessed for both the eye-wiping response and Evan's Blue extravasation (data not shown).

RTX desensitization to chemical and thermal nociception was also examined in two other standard assays—response to formalin injection into the forepaw^{6,9} and tail immersion into 50°C water.⁸ RTX blocked nociception in both cases and did so with greater potency than did capsaicin (Table 2).

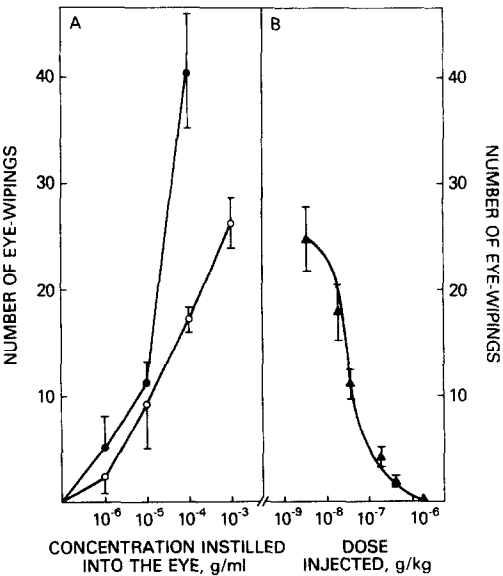


Fig. 6. Dose dependence of eye wiping in response to instillation of RTX or capsaicin. A. RTX (●) or capsaicin (○) solutions at the indicated concentrations were instilled into the eyes of rats. Each value is the mean ± S.D. for 12 animals in three experiments. B. Animals were treated by s.c. injection of RTX (▲) at the indicated doses and the eye-wiping response to 10⁻³ g/ml capsaicin was determined 6 h later. Values are the mean ± S.D. for 8–10 animals in two experiments.

DISCUSSION

Our data strongly argue that RTX functions as a capsaicin analog. RTX induced each of the three characteristic acute physiological responses to capsaicin which we examined—hypothermia, neurogenic inflammation, and pain. The acute response to RTX was followed by desensitization, a typical feature of capsaicin but not of unrelated irritants. The

Table 2. Effect of resiniferatoxin pretreatment on sensory functions

Pretreatment*	Pain index†	Latency time (s)‡
Solvent only	3.0	6.8 ± 1.3
RTX, 1 µg/paw	3.0	7.2 ± 1.3
RTX, 10 µg/paw	1.5	6.0 ± 1.0
RTX, 100 µg/paw	0.2	42.0 ± 9.3
Capsaicin, 3 mg/paw	0.2	19.4 ± 8.1

*Capsaicin and RTX at the indicated doses were injected under the plantar surface of the forepaw in a volume of 50 µl.

†Four hours after pretreatment, 50 µl of 5% formalin solution was injected into the treated paw. The behavior of the animals was observed for 1 h after the injection and the pain response was quantitated as described.⁶

‡In the same animals 8 h after pretreatment, the tail withdrawal latency time was determined.⁸ Values are the mean ± S.D. for 6–10 animals in two experiments.

RTX-desensitized animals, moreover, displayed cross-tolerance to capsaicin.

Although RTX mimicked capsaicin in its qualitative action, quantitatively it differed dramatically in potency. RTX was much more potent than capsaicin for induction of hypothermia (7×10^3 -fold, administered s.c.), for induction of neurogenic inflammation (1×10^3 -fold, applied topically), and for desensitization of neurogenic inflammation (2×10^3 -fold, administered s.c.). In our preliminary studies in the mouse, we had likewise found RTX to be 2×10^3 -fold more potent for induction of hypothermia.³

In addition to being more potent than capsaicin in the above assays, RTX also displayed a different spectrum of action. The potency of RTX in the eye wiping assay, a measure of acute pain, was within a factor of 2 of that of capsaicin, in contrast to the 10^3 – 10^5 -fold differences described above. For desensitization of neurogenic inflammation, a single dose of RTX afforded complete desensitization, whereas the maximum tolerated dose of capsaicin caused only partial desensitization. This qualitative difference may reflect different relative toxicities upon s.c. ad-

ministration. The maximum tolerated dose of RTX was approximately 100-fold its ED_{50} for desensitization of neurogenic inflammation, whereas the ED_{50} for capsaicin was the maximum tolerated dose.

Differences in pharmacokinetics may also explain some of the disparities in relative potencies. We do not know the relative rates of absorption of capsaicin and RTX across the conjunctiva or their relative rates of flushing by lacrimation, for example. Nonetheless, such factors seem unlikely to account for the differences of 10^5 -fold which we have observed.

Comparison of the potencies of RTX for desensitization in the eye-wiping and Evans Blue extravasation responses also implies heterogeneity of action. Desensitization in the eye-wiping assay was achieved with a 100-fold lower dose of RTX than in the latter assay, although desensitization with RTX was carried out in the same fashion in both cases and indeed both responses could be assessed in the same animals.

Previous investigations of capsaicin and related analogs have likewise suggested different patterns of response for different congeners. Whereas capsaicin causes both acute irritation and long lasting desensitization, zingerone, the pungent ingredient in ginger, lacks desensitizing activity.¹⁵ It had been suggested that the acylamide linkage, which is present in capsaicin but missing in zingerone, might be essential for desensitization.¹⁵ The high desensitizing potency of RTX, which lacks the acylamide linkage, argues against this suggestion.

RTX represents a powerful new tool for probing mechanisms of pain, neurogenic inflammation, and thermoregulation. Its high potency should facilitate receptor analysis; its structural relationship to the phorbol-related diterpenes may afford a new family of derivatives to define the capsaicin pharmacophore. Given the potential therapeutic implications of C-fiber desensitization, the unique spectrum of action of RTX is of particular interest.

Acknowledgement—We thank Dr Stuart H. Yuspa for critical reading of this manuscript.

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(Accepted 28 November 1988)