Course of fever response to repeated administration of sublethal doses of lipopolysaccharides, polyinosinic:polycytidylic acid and muramyl dipeptide to rabbits

D. Soszynski, W. Kozak and M. Szewczenko

Department of Physiology, Medical University of Bydgoszcz, 24 Karlowicza Str., PL - 85-092 Bydgoszcz (Poland)
Received 15 March 1990; accepted 22 June 1990

Summary. The purpose of the present study was to examine the development of tolerance to three structurally dissimilar pyrogens, i.e., lipopolysaccharide (LPS), muramyl dipeptide (MDP) and polyinosinic:polycytidylic acid (poly I:C) in rabbits. The possibility of pyrogenic cross-tolerance among these agents has also been studied. It was observed that repeated injection of sublethal doses of LPS and MDP was connected with the changing of biphasic fever to monophasic. The consequence of this was a drop in the fever index. In contrast to LPS and MDP, the repeated administration of poly I:C did not result in such changes. Successive injections of this pyrogen always evoked biphasic fever. We also demonstrated that pyrogenic cross-tolerance between LPS and MDP did not occur. The cross-tolerance between LPS and MDP did not occur. The cross-tolerance among pyrogens was possible if they originated from the same class, for example endotoxin from *Salmonella abortus* eq. and endotoxin from *Escherichia coli*.
Key words. Fever; pyrogens; tolerance; cross-tolerance; rectal temperature; rabbit.

Exogenous pyrogens, i.e., agents derived from outside the host, produce fever by their ability to induce synthesis and release of endogenous pyrogen (EP) from the host's phagocytic cells [1]. The liberated EP is considered to be an essential mediator of the action of various exogenous pyrogens in the pathogenesis of fever [2]. Fever induction and other biological functions of EP are related to mobilization of intracellular calcium which can activate phospholipase(s) [3]. This, in turn, leads to the liberation of arachidonic acid from membrane phospholipids and then its conversion to eicosanoids [4]. The weight of the available data favours PGE_2 as the most important centrally acting mediator of the fever mechanism [5].

Of the considerable number of exogenous pyrogens known, LPS is the agent most frequently used in studies of fever. MDP and poly I:C have also been used in some studies. The LPS molecule is an integral part of the cell walls of gram-negative bacteria [1]. Muramyl peptides, on the other hand, are components of the cell walls of gram-positive bacteria. Muramyl dipeptide (MDP: N-acetyl-muramyl-L-alanyl-D-isoglutamine) is the simplest chemical structure capable of substituting for killed mycobacteria in Freund's complete adjuvant [6]. It has been established that interleukin-1 (Il-1) is the endogenous factor which mediates the fever response to LPS and MDP [7, 8].

The pyrogenic poly I:C is a synthetic double-stranded RNA, commonly used as an inducer of interferon production [1]. This kind of pyrogen has been used in fever research as a model of virus infection. It is thought that interferon is a potent endogenous pyrogen, and mediates the fever response to poly I:C [9, 10].

A single intravenous injection of exogenous pyrogen results in biphasic fever. The molecular mechanisms indispensable for the induction of the first and second phases of fever are far from being sufficiently understood. Recent data suggest that the second phase is related to the release of EP, which enhances arachidonic acid turnover, whereas the first phase of fever is considered to be a result of an as yet unknown effect of the exogenous pyrogen [11]. Fever response to an exogenous pyrogen is a non-specific process. This means that irrespective of the chemical structure and origin of the exogenous pyrogen the same mechanism is involved in inducing fever. This process is invariably initiated by the synthesis and release of EP.

In studies of the mechanism of fever development some investigators have reported that repeated aministration of bacterial pyrogen at 24-h intervals usually resulted in a modification of the course of fever [12]. This has been termed pyrogenic tolerance, and in general it is characterized by the attenuation of the fever response. We presume therefore that some steps of the fever mechanism may mediate the induction of pyrogenic tolerance. In the present study we investigated the time-course of changes in rectal temperature in response to repeated administration of three structurally different exogenous pyrogens in rabbits. We also investigated the possibility of transferring the tolerance between these pyrogens, in order to address the question of whether the fever tolerance is specific to a certain pyrogen.

Materials and methods

Male New Zealand white rabbits weighing 3–5 kg were used throughout the study. The animals were housed in individual cages in an animal room at 20 °C, and had free access to food and water.

Pyrogenic substances and saline control were administered into the ear marginal vein. All glassware, needles, syringes and phosphate-buffered saline (PBS) were pyrogen-free.

The rectal temperature was measured using a precalibrated thermistor probe inserted 10 cm beyond the anus and taped to the tail. Temperature measurements were

recorded automatically using a digital temperature recorder. Recordings were performed on unrestrained animals at the ambient temperature, 20 °C.

Animals were divided into the following three experimental groups:

- in the first group (5 rabbits), pyrogenic tolerance was induced in rabbits by four daily repeated injections of LPS from *Salmonella abortus* eq. (Sigma), at a dose of 0.3 µg/kg; on the 5th day of the experiment the rabbits were injected with LPS from *Escherichia coli* (Kroeger 08, Biomed) at a dose of 0.3 µg/kg, and on the 6th day with MDP (Daiichi Pharmaceutical Co.) at a dose of 100 µg/kg;
- in the second group (5 rabbits), pyrogenic tolerance was induced in animals by four consecutive daily injections of MDP (100 µg/kg); on the 5th day of the experiment rabbits were injected with LPS *S. abortus* eq. (0.3 µg/kg);
- in the third group (5 rabbits), pyrogenic tolerance was induced by five consecutive daily injections of poly I:C (Sigma) at a dose of 50 µg/kg; on the 6th day the rabbits were injected with LPS *S. abortus* eq. (0.3 µg/kg), and on the 7th day with MDP (100 µg/kg).

The temperature of each rabbit was measured and recorded for 30 min before the pyrogen injection and for 6 h afterwards.

Changes of the rectal temperature (ΔT) are expressed as a deviation from the base line recorded at the time of injection.

The integrated area under the temperature/time curves, the fever index, was expressed in °C · h (for 6 h).

Statistical analysis of the data was performed using Student's t-test. Data are expressed as mean ± SD of the mean.

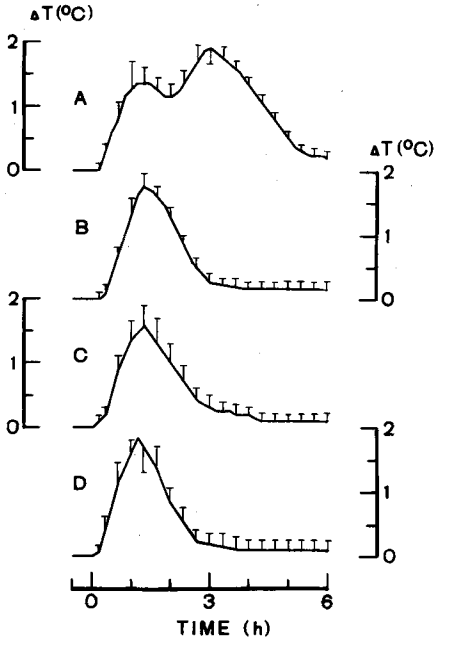


Figure 1. Changes (mean ± SD, n = 5) of the rectal temperature (ΔT) in rabbits during induction of tolerance to LPS (*S. abortus* eq., 0.3 µg/kg). Respective days of tolerance are as follows: first (*A*); second (*B*); third (*C*); fourth (*D*). Pyrogen was injected at 0 min.

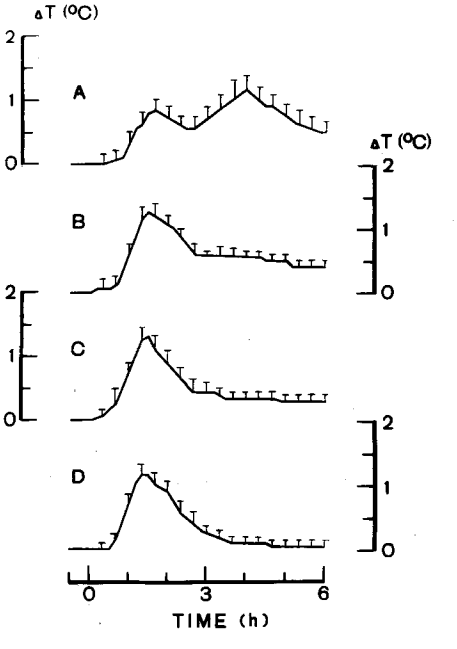


Figure 2. Changes (mean ± SD, n = 5) of the rectal temperature (ΔT) in rabbits during induction of tolerance to MDP (100 µg/kg). Successive days of tolerance are described as in fig. 1. Pyrogen was injected at 0 min.

Results

Induction of tolerance to exogenous pyrogens. To examine the development of pyrogenic tolerance to LPS *S. abortus* eq., the rabbits were injected intravenously with LPS for four successive days. As shown in figure 1, significant tolerance to LPS was observed. After administration of the first dose of LPS a biphasic rise of temperature was observed. The following injections of the same doses of LPS at 24-h intervals evoked only a monophasic rise of temperature. A significant decrease of the fever index was observed as early as the second day of the experiment ($p < 0.05$), and the following injections did not change it markedly (fig. 3).

MDP at a dose of 100 µg/kg produced biphasic fever (fig. 2). Comparing the doses used, MDP was less pyrogenic than LPS. As shown in fig.2, animals made tolerant to MDP produced a monophasic rise of temperature. Unlike the situation with LPS, however, we observed a gradual decrease of the fever index in response to repeated administration of MDP. The significant reduction in febrile responses to this pyrogen was not seen before the 4th day of the experiment. There was a significant decrease of the fever index between the first and the fourth day of induction of tolerance to MDP ($p < 0.01$) (fig. 3). In the case of repeated administration of poly I:C such changes were not observed. Successive injections of this pyrogen always evoked a biphasic rise of rectal tempera-

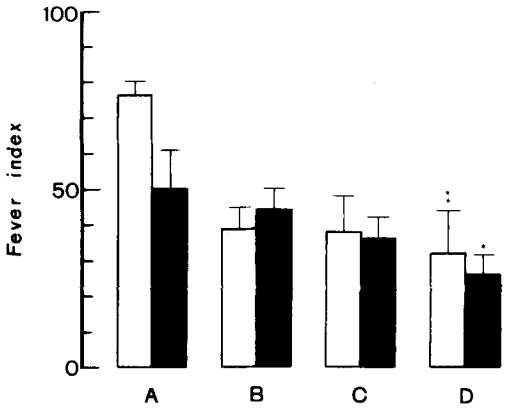


Figure 3. Comparison of fever indexes (mean ± SD, n = 5) during repeated administration of LPS (*S. abortus* eq., 0.3 µg/kg) (open bars) and MDP (100 µg/kg) (closed bars). For symbols see fig. 1. * and ** significantly different from first injections of MDP ($p < 0.01$) and LPS ($p < 0.01$), respectively.

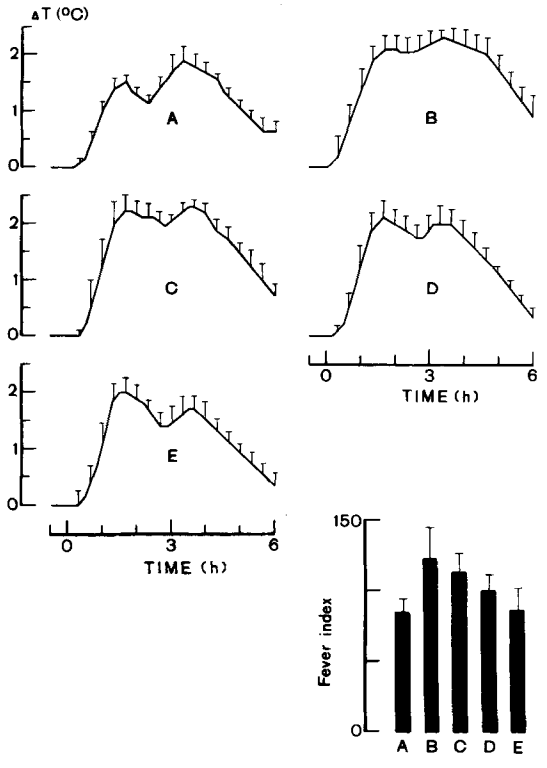
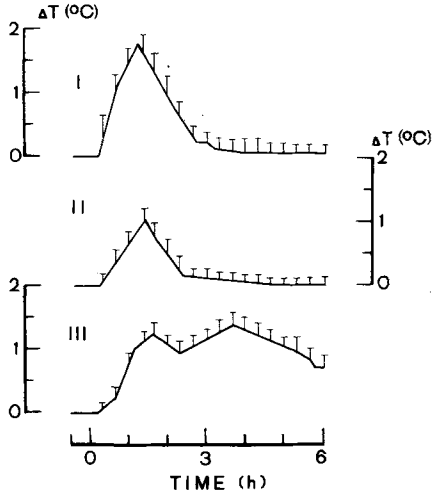


Figure 4. Changes of the rectal temperature (ΔT) and fever indexes in rabbits during five daily injections of poly I:C (50 µg/kg). Consecutive days of experiment are indicated as follows: first (*A*); second (*B*); third (*C*); fourth (*D*); fifth (*E*). Pyrogen was injected at 0 min. Mean ± SD, n = 5.



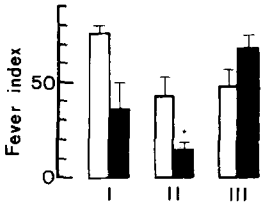
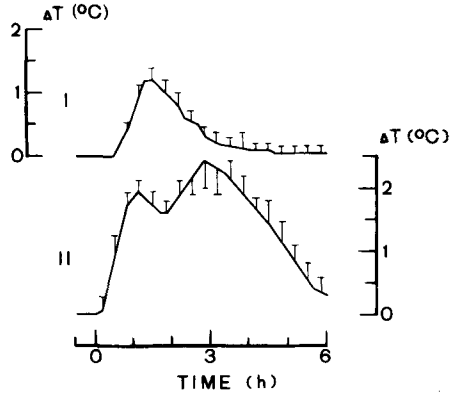


Figure 5. Changes (mean ± SD, n = 5) of the rectal temperature (ΔT) and fever indexes (closed bars) in rabbits on the fourth day of induction of tolerance to LPS (*S. abortus* eq., 0.3 µg/kg) (I) followed by injection of LPS (*E. coli*, 0.3 µg/kg) (II) and MDP (100 µg/kg) (III) on the fifth and the sixth days of the experiment, respectively. Open bars represent the fever indexes of fresh rabbits in response to these pyrogens. Pyrogen was injected at 0 min. * significantly different from control value ($p < 0.001$).



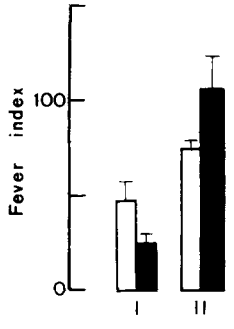


Figure 6. Changes (mean ± SD, n = 5) of the rectal temperature (ΔT) and fever indexes (closed bars) in rabbits on the fourth day of induction of tolerance to MDP (100 µg/kg) (I) and after injection of LPS (*S. abortus* eq., 0.3 µg/kg) (II) on the fifth day of experiment. Open bars represent the fever indexes of fresh rabbits in response to these pyrogens. Pyrogen was injected at 0 min.

ture (fig. 4). An increased value of the fever index was noticed on the second day of the experiment. During the following days, the fever index showed a stepwise decrease to the level of the first day.

Induction of pyrogenic cross-tolerance. Intravenous injection of LPS *E. coli* (0.3 µg/kg) evoked a monophasic rise of rectal temperature in rabbits tolerant to LPS from *S. abortus* eq. On the other hand, animals which had been made tolerant to LPS from *S. abortus* eq. responded with biphasic fever to MDP administration (fig. 5). Similarly, the rabbits tolerant to MDP which were given endotoxin afterwards developed the biphasic fever characteristic for fresh animals (fig. 6).

These results suggest the absence of pyrogenic cross-tolerance between LPS and MDP. Repeated administration of poly I:C did not interfere with fever responses to LPS and MDP given afterwards (data not shown).

Discussion

The results of this study show that fever indexes were decreased significantly during induction of tolerance to LPS and MDP. Analysis of changes of temperature as a function of time indicates that the decrease of the fever index accompanying the reduction of tolerance to these agents (fig. 3) is related to a reduction of the second phase of fever (figs. 1, 2). The latency of fever onset after consecutive injections of pyrogen remained unchanged. The first peak of rectal temperature during repeated administration was reached at the same time. In the period of 3 h after injection the rectal temperature approximated to the normal temperature.

We assume that the changes in time-course of the rectal temperature and fever indexes described above are concomitant with tolerance-induction and are characteristic for this process. However, the changes were only observed in experiments with LPS and MDP. In contrast to this, repeated daily injections of the same dose of poly I:C invariably induced biphasic fever (fig. 4). This suggests that, at least during the period of the experiment, the rabbits did not become tolerant to this pyrogen.

The convergence of the time-courses of tolerance to LPS and MDP, as well as the non-specific character of fever in general, might suggest the possibility of transferring tolerance between these pyrogens. It became apparent, however, that cross-tolerance among pyrogens was only possible if they were of the same class, eg. endotoxin from *S. abortus* eq. and endotoxin from *E. coli* (fig. 5), indicating that tolerance has a specific character.

With respect to the course of fever it is possible to conclude that the specificity of tolerance is related to the second phase of fever, since the first phase was observed instantly, both during induction of tolerance and in cross-tolerance experiments. It is thought that EP is responsible for induction of the second phase of fever. Reduction of this phase in tolerant rabbits may suggest either (i) the inhibition of EP synthesis or (ii) adaptive changes in the reactivity of EP-producing cells to exogenous pyrogens. With respect to the former, it has been demonstrated in in vitro studies that macrophages isolated from tolerant animals revealed an unchanging capacity for EP production[1]. The biphasic fever observed in our experiments during cross-tolerance studies using different classes of exogenous pyrogens seems to be consistent with this. This observation, together with the fact that there was no second phase of fever in cross-tolerance experiments using a pyrogen that was structurally the same, suggests that each class of pyrogen can stimulate its specific receptor-like and adaptable route for EP production.

Available experimental data have provided evidence for the existence of receptors for exogenous pyrogens on membranes of immunocompetent cells[13–15]. It is possible, therefore, that both the induction of tolerance and the quenching of EP synthesis suggested here are the result of a decrease in the accessibility of receptors specific for certain pyrogens. Glucocorticoids are potent endogenous factors which can modulate receptor accessibility[16,17]. It has been found that during induction of tolerance the blood level of these hormones increases[18,19]. The physiological significance of such an adaptation may be related to protection of the host against hyperreactivity of its defence system to certain antigens, whilst maintaining the reactivity to another pyrogen.

Lack of tolerance to poly I:C suggests that this pyrogen may generate fever through mechanisms which do not interfere with the adaptive changes observed during induction of tolerance to LPS and MDP. Fever induced by all three of these structurally different pyrogens is abolished by non-steroidal antipyretics which can inhibit prostaglandin synthesis[5]. This suggests that the different characteristics of poly I:C fever may relate to earlier steps in the fever-inducing mechanism, i.e., at the level of receptor signalling or EP production. It is as yet uncertain whether the EP is a single protein or represents a group of fever-inducing proteins. Available evidence attributes the ability to act as an endogenous pyrogen not only to Il-1, but also to IFN[9], tumor necrosis factor[20], and interleukin-6[21].

Acknowledgment. The authors thank Prof. Shozo Kotani for supplying MDP for this study.

1 Dinarello, C. A., and Wolff, S. M., in: Handb. exp. Pharmac. 60: Pyretics and Antipyretics, pp. 73–112. Ed. A. S. Milton. Springer-Verlag, Berlin 1982.
2 Hellon, R., and Townsend, Y., Pharmac. Ther. *19* (1983) 211.
3 Kozak, W., Milton, A. S., Abul, H., Davidson, J., and Rotondo, D., Cell. Signaling *4* (1989) 345.
4 Bernheim, H. A., Yale J. biol. Med. *59* (1986) 151.
5 Milton, A. S., in: Handbook of Experimental Pharmacology. 60: Pyretics and Antipyretics, pp. 257–303. Ed. A. S. Milton. Springer-Verlag, Berlin 1982.
6 Kotani, S., Azuma, I., Takada, H., Tsujimoto, M., and Yamamura, Y., in: Biological Response Modifiers in Human Oncology and Immunology, pp. 117–158. Eds T. Klein, S. Specter, H. Friedman and A. Szentivanyi. Plenum, New York 1983.
7 Dinarello, C. A., and Krueger, J. M., Fedn Proc. *45* (1986) 2545.
8 Dinarello, C. A., Rev. infec. Dis. *6* (1984) 51.

9 Won, S. J., and Lin, M. T., Am. J. Physiol. *23* (1988) 499.
10 Dinarello, C. A., Bernheim, H. A., Duff, G. W., Le, H. V., Nagabhushan, T. L., Hamilton, N. C., and Coceani, F., J. clin. Invest. *74* (1984) 906.
11 Bligh, J., in: Handbook of Experimental Pharmacology. 60: Pyretics and Antipyretics, pp. 25–71. Ed. A. S. Milton. Springer-Verlag, Berlin 1982.
12 Dinarello, C. A., Elin, R. J., and Chedid, L., J. infec. Dis. *138* (1978) 760.
13 Silverman, D. H. S., Krueger, J. M., and Karnovsky, M. L., J. Immunol. *136* (1986) 2195.
14 Wright, S. D., and Jong, M. T. C., J. exp. Med. *164* (1986) 1876.
15 Torrence, P. F., and De Clercq, E., in: Handbook of Experimental Pharmacology. 71: Interferons and their Applications, pp. 233–258. Eds P. E. Came and W. A. Carter. Springer-Verlag, Berlin 1984.
16 Wielckens, K., Deles, T., Muth, A., Freese, V., and Kleeberg, H.-J., J. Steroid Biochem. *27* (1987) 413.
17 Zor, U., Ostfeld, J., Talmon, J., and Lahar, Y., in: Advances in Prostaglandin, Tromboxane, and Leukotriene Research, pp. 519–523. Eds B. Samuelsson, P. Y.-K. Wong and F. F. Sun. Raven Press, New York 1989.
18 Suzuki, T., Jap. J. Bact. *15* (1960) 294.
19 Besedovsky, H., Sorkin, E., Keller, M., and Miller, J., Proc. Soc. exp. Biol. Med. *150* (1975) 466.
20 Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, J. S., Palladino, M. A. Jr, and O'Connor, J. V., J. exp. Med. *163* (1986) 1433.
21 Durum, S. K., and Oppenheim, J. J., in: Fundamental Immunology, 2nd edn, pp. 639–661. Ed. W. E. Paul. Raven Press, New York 1989.

0014-4754/91/010043-05$1.50 + 0.20/0

Discharge pattern analysis suggests existence of a low-threshold calcium channel in cold receptors

K. Schäfer, H. A. Braun[a] and L. Rempe

Institut für Zoophysiologie, Universität Hohenheim, Postfach 700 562, D-7000 Stuttgart 70, and [a] Institut für Physiologie, Universität Marburg, Deutschhausstr. 1, D-3550 Marburg (Federal Republic of Germany)
Received 20 March 1990; accepted 19 June 1990

Summary. The regular periodic activity patterns of mammalian cold receptors have been quantitatively studied. Analysis of the timing of either single impulses or impulse groups demonstrated that the periodic receptor process is maintained independently of impulse generation and continues to operate under conditions when afferent impulses are not initiated. These results imply that the underlying conductances must be operational at threshold potentials related to impulse generation. In addition to temperature, the periodic process is considerably sensitive to calcium, which affects mainly the probability of impulse generation during each cycle. Reduction of external calcium and application of calcium entry blockers with relative selectivity for low-threshold calcium channels are similarly effective in modulating cold receptor activity. The data imply the existence of a low-threshold calcium conductance at the sensory terminal.
Key words. Cold receptor; periodic discharge pattern; transducer process; low-threshold calcium channel.

Relatively little is known about the nature of the cellular processes which in cold receptors convert patterns of heat energy into afferent neuronal signals. These sensors are considered to be 'free' nerve endings [1] and their small size and low distribution density have so far not allowed us to study their transducer processes directly. However, periodic components have been observed in the temporal pattern of afferent activity in all mammalian cold receptor populations [2]. Several studies provide evidence that a temperature- and calcium-sensitive receptor potential oscillation generates afferent impulses when it exceeds a threshold value [3–5]. Since the stimulating effect of reduced external calcium can be exactly mimicked by menthol [6], which selectively impairs calcium channel conductance [7, 8], the existence of a specific calcium channel at the sensory terminal has been postulated [6, 9]. Menthol interferes with calcium entry through two of the calcium channels present in dorsal root ganglion (DRG) cells [7]. These conductances differ in their physical and pharmacological characteristics [10, 11]. Here we present evidence that the receptor potential oscillation is maintained at threshold potentials related to impulse generation, indicating the existence of a low-threshold channel. This view is supported by the observation that calcium entry blockers affecting preferentially low-threshold channels are effective in modulating cold receptor activity.

Material and methods

Isolated preparations of the tongues of cats were prepared and perfused as previously described [12]. The normal perfusing medium contained 118.40 mM NaCl, 26.60 mM $NaHCO_3$, 2.83 mM KCl, 1.32 mM KH_2PO_4, 1.46 mM $MgSO_4$, 1.53 mM $CaCl_2$, and 11.60 mM glucose. In some experiments, the calcium concentration was changed to 0.5 mM. Calcium channel modulators were applied by addition to the perfusing medium. The lingual nerve was dissected into fine strands, which were placed on a platinum electrode for the recording of single unit activity. Cold receptors were stimulated by a water-