Effects of \(\omega\)-Agatoxin IVA, a P-Type Calcium Channel Antagonist, on the Development of Spinal Neuronal Hyperexcitability Caused by Knee Inflammation in Rats

JOHANNES NEBE, ANDREA EBERSBERGER, HORACIO VANEGAS, AND HANS-GEORG SCHAIBLE

Physiologisches Institut der Universität Würzburg, D-97070 Würzburg, Germany

Nebe, Johannes, Andrea Ebersberger, Horacio Vanegas, and Hans-Georg Schaible. Effects of \(\omega\)-agatoxin IVA, a P-type calcium channel antagonist, on the development of spinal neuronal hyperexcitability caused by knee inflammation in rats. J. Neurophysiol. 81: 2620–2626, 1999. Both N- and P-type high-threshold calcium channels are located presynaptically in the CNS and are involved in the release of transmitters. To investigate the importance of P-type calcium channels in the generation of inflammation-evoked hyperexcitability of spinal cord neurons, electrophysiological recordings were made from wide-dynamic-range neurons with input from the knee joint in the anesthetized rat. The responses of each neuron to innocuous and noxious pressure onto the knee and the ankle were continuously assessed before and during the development of an inflammation in the knee joint induced by the injections of K/C into the joint cavity. The specific antagonist at P-type calcium channels \(\omega\)-agatoxin was administered into a 30-\(\mu\)l trough on the spinal cord surface above the recorded neuron. In most neurons the application of \(\omega\)-agatoxin before induction of inflammation slightly enhanced the responses to pressure onto the knee and ankle or left them unchanged. Two different protocols were then followed. In the control group (13 rats) only Tyrode was administered to the spinal cord during and after induction of inflammation. In these neurons the responses to mechanical stimuli applied to both the inflamed knee and to the noninflamed ankle showed a significant increase over 4 h. In the experimental group (12 rats) \(\omega\)-agatoxin was applied during knee injection and in five 15-min periods up to 180 min after kaolin. This prevented the increase of the neuronal responses to innocuous pressure onto the knee and to innocuous and noxious pressure onto the ankle; only the responses to noxious pressure onto the knee were significantly enhanced during development of inflammation. Thus the development of inflammation-evoked hyperexcitability was attenuated by \(\omega\)-agatoxin, and this suggests that P-type calcium channels in the spinal cord are involved in the generation of inflammation-evoked hyperexcitability of spinal cord neurons. Finally, when \(\omega\)-agatoxin was administered to the spinal cord 4 h after the kaolin injection, i.e., when inflammation-evoked hyperexcitability was fully established, the responses to innocuous and noxious pressure onto the knee were reduced by 20–30% on average. The shift in the effect of \(\omega\)-agatoxin, from slight facilitation or no change of the responses before inflammation to inhibition in the state of hyperexcitability, indicates that P-type calcium channels are important for excitatory synaptic transmission involved in the maintenance of inflammation-evoked hyperexcitability.

**INTRODUCTION**

In neurobiological research on nociception and pain, the functional role of diverse types of ion channels in nociceptive neurons has recently elicited considerable interest. Because neuronal calcium channels play a critical role in the release of neuromediators and in somatodendritic excitability (Bertolino and Llinas 1992; Miljanich and Ramachandran 1995; Olivera et al. 1994; Tsien 1993), their participation in the mechanisms of acute and persistent nociception has become a focus of recent investigations. Electrophysiological, pharmacological, and molecular genetic techniques have identified high-threshold voltage-dependent calcium channels (VDCCs) of the L, N, P, Q or P/Q, and R type in central neurons (Miljanich and Ramachandran 1995; Olivera et al. 1994; Tsien 1993).

P-type VDCCs are located in presynaptic neuronal terminals, and they are involved in the release of the transmitters glutamate, aspartate, dopamine, serotonin, norepinephrine, GABA, and probably glycine and presumably others (Kimura et al. 1995; Miljanich and Ramachandran 1995; Takahashi and Momiyama 1993; Turner et al. 1992, 1993). The potent and selective antagonist of P-type calcium channels \(\omega\)-agatoxin IVA (AgaIVA) can be used to identify P-type-calcium–dependent synaptic and other cellular processes. AgaIVA has been shown to block excitatory (Castillo et al. 1994; Luebke et al. 1993; Yamamoto et al. 1994) as well as inhibitory synaptic transmission (Takahashi and Momiyama 1993). P-type channels may sustain pre- as well as postsynaptic calcium currents (Igelmund et al. 1996).

Behavioral and electrophysiological experiments showed an involvement of P-type calcium channels in spinal nociceptive processing under particular conditions. In awake rats, intrathecal administration of AgaIVA decreased the late phase of nociceptive behavior in the formalin test (Malmberg and Yaksh 1994). The development of secondary hyperalgesia and allodynia after the intradermal injection of capsaicin (Sluka 1997) or the induction of an inflammation in the knee joint (Sluka 1998) could be prevented by the application of AgaIVA through a microdialysis fiber implanted in the spinal dorsal horn. In recordings from spinal cord neurons, AgaIVA reduced the discharges of nociceptive neurons in the late phase of the formalin response (Diaz and Dickenson 1997). In spinal cord neurons with input from the knee joint, AgaIVA administration to the spinal cord caused on average a slight facilitation of dorsal horn neuronal responses to innocuous and noxious pressure applied to a normal knee; however, in rats in which the knee had been inflamed for a few hours with kaolin/carrageenan, AgaIVA reduced the responses to innocuous and noxious pressure by \(\sim 27\%\) (Nebe et al. 1997). These findings
suggest that P-type VDCC-dependent processes are involved in the spinal cord processing of input from inflamed tissue.

We addressed the question whether P-type VDCCs are involved in the generation of inflammation-evoked hyperexcitability of spinal cord neurons. Because our previous study (Nebe et al. 1997) was done in one population of rats with normal knees and in another population with one knee inflamed, information could only be obtained regarding the involvement of P-type channels in the maintenance but not in the generation of inflammation-evoked hyperexcitability. The latter process requires the intraspinal release of several transmitters and neuromodulators such as excitatory amino acids and the neuropeptides substance P, neurokinin A, and calcitonin gene-related peptide and probably others and the activation of the corresponding receptors (for references see Neugebauer et al. 1993–1995, 1996a,b). These systems are likewise involved in other models of persistent nociception (cf. Coderre et al. 1993; Dubner and Ruda 1992; Willis 1994).

If P-type VDCCs are involved in the release of transmitters and modulators that are important for the generation of inflammation-evoked hyperexcitability, then the application of AgaIVA should have an influence on the progressive increase of neuronal responses that is seen during the development of inflammation (Schaible and Grubb 1993). We therefore administered AgaIVA to the spinal cord before and during the induction of knee inflammation and studied whether this treatment would interfere with the central sensitization process that is usually seen under these conditions.

METHODS

Preparation

Experiments were performed on 25 male Wistar rats (280–440 g) anesthetized with sodium thiopentone (Trapanal, BYK, initial dose 85–115 mg/kg ip). The trachea was cannulated, and catheters were inserted into the common carotid artery and the external jugular vein. The animals breathed spontaneously, and a gentle jet of oxygen was blown toward the opening of the tracheal cannula. Body temperature was kept at 37°C by means of a feedback-controlled system. Additional injections of thiopentone (20–50 mg/kg ip) were given as necessary to achieve a sufficiently deep level of anesthesia as assessed by the absence of corneal or leg withdrawal reflexes. Mean arterial blood pressure was stable at 90–120 mmHg. Spinal cord segments L1–L4 were exposed by laminectomy. The dura mater was opened, and the threshold within receptive fields were determined with the absence of corneal or leg withdrawal reflexes. Mean arterial blood pressure was stable at 90–120 mmHg. Spinal cord segments L1–L4 were exposed by laminectomy. The dura mater was opened, and the threshold within receptive fields were determined with

Recording and sampling of neurons

By using glass-insulated carbon filaments for recording, individual neurons were identified by spike shape and height. The action potentials were continuously monitored on a digital oscilloscope and recorded on videotape. The signal was also fed into a window discriminator, the output of which was processed by an A/D interface and a personal computer so that peristimulus time histograms could be constructed. Dorsal horn neurons were chosen for study if they responded to pressure applied to the ipsilateral left knee but did not respond to brushing or squeezing of the skin over the knee. The size of and the threshold within receptive fields were determined with stimulation of the skin (brushing, squeezing of skin folds with forceps) and of the deep tissue (compression of joints and muscles). Only neurons were studied that responded to innocuous mechanical stimuli and showed stronger responses to noxious mechanical stimuli (wide-dynamic-range neurons). Mechanical test stimuli of two standard intensities were then applied to the knee and to the ankle. A calibrated mechanical device was used for compression of the knee joint in the mediolateral axis; for innocuous intensity, a 1.9 N/40 mm² holding pressure was applied, and for noxious intensity (felt painful when applied to the experimenter’s fifth finger) the knee was compressed with either 5.9 or 7.8 N/40 mm². Two clips were used for stimulation at the ipsilateral ankle (1.1 N/20 mm² for innocuous, 5.8 N/20 mm² for noxious stimulation). Each pressure stimulus lasted for 15 s. Each neuron was continuously recorded before and through the development of knee inflammation.

Experimental protocol

Innocuous and noxious test stimuli were applied sequentially to the knee and then to the ankle; this sequence was repeated every 3–5 min, even when the manipulations described subsequently were being carried out. In 13 control rats (the Tyrode group, see Fig. 1A), after the
neuronal baseline responses were established during 1 h, the Tyrode
solution was removed from the trough, and 20 μl of 0.1 μM AgaIVA
(either a gift from Pfizer or purchased from Peninsula) in 165 mM
NaCl, pH 7.0, was delivered to the trough with an Eppendorf pipetter.
AgaIVA remained in the trough for 30 min (Fig. 1), and then the
trough was rinsed three times and refilled with Tyrode. Thirty minutes
later, an experimental inflammation was induced in the left knee joint.
With this purpose a 27-gauge needle was introduced through the
patellar ligament, and 70 μl of a 4% kaolin suspension was slowly
injected into the articular cavity. Then the joint was slowly flexed and
extended for 15 min. Thereafter, 70 μl of a 2% carrageenan solution
was injected, and the joint was moved for another 5 min. Forty-five
minutes after the kaolin injection, a period began in which Tyrode was
exchanged in the trough every 15 min up to 180 min after kaolin. One
hour after the last Tyrode change, a final application of AgaIVA of
30-min duration was made (Fig. 1). In another 13 rats (the AgaIVA-
group, see Fig. 1B), the influence of AgaIVA on the increases of
responses to mechanical stimuli during the development of inflam-
amation was assessed. The protocol was similar as in the Tyrode group
except that 1) the first AgaIVA application lasted for 1 h and the
kaolin/carrageenan injection was performed in the middle of this
period (Fig. 1B) and 2) between 45 and 180 min after kaolin AgaIVA
and Tyrode were alternated in the trough every 15 min (Fig. 1B).

Quantification of results

The responses to pressure stimuli were calculated by subtracting the
ongoing activity in the preceding 15 s (if any) from the total activity
during an innocuous or a noxious test stimulus. To establish the
predrug baseline of a neuron, ~10 test responses to each type of
stimulus were averaged and set to 0. The responses of the neuron to
mechanical stimuli after the induction of inflammation were grouped
in the following time intervals: baseline before the first application of
AgaIVA, during the 30 min of the first application of AgaIVA, 30–90
min, 90–150 min, 150–210 min, and 210–240 min after kaolin. The
intraarticular injection of kaolin was taken as minute 0, and an average
value was calculated of the responses to innocuous or noxious stim-
ulation in each of these time intervals. The baseline average was set
to 0, and all other average values were compared with it (see Fig. 2).
Wilcoxon matched-pairs signed rank test was used to analyze inflam-
amation-evoked changes of responses within the treatment groups. The
Mann-Whitney U test was used to compare the mean values of

![Graph showing changes of the responses of continuously recorded spinal cord neurons to pressure stimuli during development of inflammation in the knee and ankle.](http://jn.physiology.org/)

**FIG. 2.** Changes of the responses of continuously recorded spinal cord neurons to pressure onto the knee (left panel) and onto the ankle (right panel) during development of inflammation in the knee. ○ neurons from experiments in which only Tyrode was adminis-
tered to the spinal cord during development of inflammation; ● neurons from experiments in which AgaIVA was adminis-
tered to the spinal cord during development of inflammation. Average baseline responses before inflam-
ination were set to 0. The values of the subsequent time intervals show the average differences between the responses of the postinjection time periods and the base-
line before inflammation. The values are expressed as mean ± SE. *P < 0.05 for the difference between
Tyrode- and AgaIVA-treated groups in the correspond-
ing time interval.

**RESULTS**

In 25 rats recordings were performed from 26 neurons with
input from the ipsilateral knee joint (in 1 experiment 2 neurons
were monitored simultaneously). The neurons were located in
segments L1–L3 at depths of 353–1136 μm (809 ± 194 μm, mean ± SD). The large majority of neurons was thus in the
deep dorsal horn. All recorded cells were wide-dynamic-range
neurons. Only 4 of 26 neurons showed ongoing discharges
before inflammation. The neurons were activated by pressure
onto the knee and other deep tissues of the leg (e.g., the ankle).
Most of the neurons had also cutaneous receptive fields on the
leg but not in the skin overlying the knee.

**Effect of AgaIVA on the development of inflammation-
induced hyperexcitability**

**MECHANICAL STIMULATION OF THE KNEE JOINT.** Figure 1 shows
results from two dorsal horn neurons from two experiments. The
graphs display the responses to innocuous and noxious
pressure applied to the knee joint before inflammation and
during the development of inflammation that was induced by
the intraarticular injections of kaolin and carrageenan (K/C). Before induction of inflammation, AgaIVA was administered
topically to the spinal cord for a period of 30 min. In this period
neither of these neurons showed a consistent change of the
responses to innocuous and noxious pressure during the ad-
ministration of AgaIVA (see time blocks before K/C in Fig. 1).
After testing the effect of AgaIVA before inflammation, K/C
were injected into the knee for induction of inflammation, and
which AgaIVA was administered to the spinal cord during the pressure. In the sample of 13 neurons from experiments in the knee, and 10 neurons also exhibited an increase to noxious showed an increase of the responses to innocuous pressure onto during development of inflammation (cf. Fig. 1 in which only Tyrode was administered to the spinal cord.

Changes of the responses to innocuous and noxious pressure were monitored. In the control neuron in Fig. 1A, fresh Tyrode solution was administered to the spinal cord during the time blocks indicated. In the neuron in Fig. 1B, AgaIVA was left on the cord for 30 min after the injection of kaolin (initial phase of the induction of inflammation) and was also administered for several periods of 15 min between 45 and 180 min after injection of kaolin (narrow rectangles). In both neurons the responses to innocuous and noxious pressure applied to the knee showed an increase during development of inflammation, although the relative changes in the AgaIVA-treated neuron were less pronounced. In both neurons, AgaIVA was administered to the spinal cord during the development of inflammation, 8 neurons showed an increase of the responses to innocuous and noxious pressure, whereas 5 neurons exhibited rather decreases of the responses. In both groups of experiments, the diameter of the injected knee joint showed a similar increase (from 55 to 59 mm in the Tyrode control group and from 56 to 61 mm in the rats in which AgaIVA was administered to the spinal cord during development of inflammation).

Figure 2, left panel, summarizes the average changes of the responses to innocuous (top) and noxious pressure (bottom) onto the knee during development of inflammation in all neurons from Tyrode control experiments (○) and from experiments in which AgaIVA was administered in the course of inflammation (●), irrespective of whether individual neurons showed increases or decreases of the responses. The average baseline responses before inflammation were set to zero. The values of the subsequent time intervals show the average differences between the responses of the postinjection time periods and the baseline before inflammation. When the responses before inflammation were compared with those in the period 210–240 min (i.e., 4 h) after induction of inflammation, the increase was found to be significant for both innocuous and noxious pressure in the Tyrode-treated control group (Wilcoxon matched-pairs signed rank test). The corresponding analysis in the AgaIVA-treated group revealed a significant increase of the responses to noxious pressure but not of the responses to innocuous pressure. Although the response increases of the AgaIVA-treated group were smaller than those of the Tyrode-treated group, the difference between groups in each time period did not reach statistical significance (Mann-Whitney U test).

In control experiments in which only Tyrode was administered to the spinal cord during development of inflammation, 9 of 10 neurons that had ankle input showed an increase of the responses to innocuous and noxious pressure. In the sample of nine neurons from experiments in which AgaIVA was administered to the spinal cord during the development of inflammation, only two neurons showed an increase of the responses to innocuous pressure, and three neurons exhibited an increase of the responses to noxious pressure.

Figure 2, right panel, shows the average changes of responses to mechanical stimulation of the ankle in all 10 neurons from control rats with ankle input (○) and in all 9 neurons with ankle input from AgaIVA-treated rats (●). The neurons from control experiments showed on average significant increases of the responses to innocuous and noxious pressure during inflammation in the knee joint. The comparison between the baseline before knee inflammation and the period 210–240 min (i.e., 4 h) after kaolin revealed a significant
The current experiments showed that the administration of AgaIVA to the spinal cord during the development of inflammation of the knee joint influenced the generation of hyperexcitability of dorsal horn neurons. When only Tyrode was administered to the spinal cord during the development of inflammation, the responses to mechanical stimuli applied to the injected knee and to the noninjected ankle showed a significant increase. By contrast, the changes of the responses were smaller in rats in which AgaIVA was applied to the spinal cord during development of inflammation. Indeed in these rats the increase of the responses to noxious pressure onto the knee showed a significant increase, whereas the responses to innocuous pressure onto the knee and the responses to pressure onto the ankle did not change significantly. The data suggest therefore that P-type calcium channels in the spinal cord are involved in the generation and development of inflammation-evoked hyperexcitability of spinal cord neurons. Furthermore, these experiments showed that the effect of a 30-min application of AgaIVA on the responses of one and the same neuron to knee stimulation switches from inconsistent to inhibitory when an inflammation develops in the knee. Thus P-type VDCCs seem to be involved in the maintenance of established hyperexcitability.

As in the previous study, AgaIVA was administered at a dose that was found in in vitro studies to be specific for the blockade of P-type VDCCs (for references see Nebe et al. 1997). AgaIVA was not continuously administered to the spinal cord but for periods of 15–30 min. This approach resembles the protocol that we had used before to study the effect of antagonists at glutamate and neuropeptide receptors on the development of hyperexcitability (Neugebauer et al. 1993, 1995, 1996a,b). The protocol of repeated applications has the advantage that the spinal cord is exposed to relatively reproducible dosages throughout the experiments. The intervals between the AgaIVA blocks allowed to test whether AgaIVA had immediate effects on the responses. Because this was not the case, all values in the defined time periods were averaged to determine the mean responses during these periods.

The features of the development of hyperexcitability, i.e., an increase of the responses to mechanical stimulation of the injected knee as well as an increase of the responses to mechanical stimulation of the noninflamed ankle, are in line with the results of our previous studies (Neugebauer et al. 1993–1995, 1996a,b; Schaible and Grubb 1993). However, the effects of inflammation on the spinal cord neurons seem to be quantitatively smaller in experiments in which a trough is injected knee as well as an increase of the responses to mechanical stimulation of the noninflamed ankle, are in line with the results of our previous studies (Neugebauer et al. 1993–1995, 1996a,b; Schaible and Grubb 1993). However, the effects of inflammation on the spinal cord neurons seem to be quantitatively smaller in experiments in which a trough is injected knee as well as an increase of the responses to mechanical stimulation of the noninflamed ankle, are in line with the results of our previous studies (Neugebauer et al. 1993–1995, 1996a,b; Schaible and Grubb 1993). However, the effects of inflammation on the spinal cord neurons seem to be quantitatively smaller in experiments in which a trough is
results from a sensitization of both primary afferent and spinal cord nociceptive neurons. The increase in the responses to stimulation of the inflamed knee most likely constitutes the neuronal basis of primary hyperalgesia, whereas the increase in the responses of the neurons to stimulation of the noninflamed ankle may correspond to the development of secondary hyperalgesia that is observed outside of the inflamed or injured area (Coderre et al. 1993; Dubner and Rudy 1992; McMahon et al. 1993; Schaible and Grubb 1993; Willis 1994).

Changes of the responses to mechanical stimulation of the inflamed knee were not totally prevented by AgaIVA. When hyperexcitability was established the administration of AgaIVA consistently reduced the responses to stimulation of the knee on average by 20–30%. The latter effect is within the range of P-type VDCC contribution in various physiological processes (Brown et al. 1994; Mintz et al. 1992; Rusin and Moises 1995; Turner et al. 1993). Thus P-type VDCCs seem to be involved in the generation and maintenance of primary hyperalgesia after joint inflammation, but on the other hand AgaIVA had only limited effectiveness. This is best explained by the fact that N-type VDCCs significantly contribute to the nociceptive synaptic processing and the generation of hyperexcitability (Diaz and Dickenson 1997; Nebe et al. 1998; Neugebauer et al. 1996c; Sluka 1998) and that the effect of different types of VDCCs is additive (Nebe et al. 1997).

By contrast, the responses to mechanical stimulation of the ankle did not show increases during development of inflammation in the knee when AgaIVA was administered to the spinal cord. Thus P-type VDCCs seem to be critically involved in the generation of secondary hyperalgesia after inflammation. These results are in general accordance with behavioral studies that showed that secondary heat hyperalgesia and allodynia after the induction of inflammation in the knee joint could be prevented by the application of AgaIVA through a microdialysis fiber implanted in the spinal dorsal horn (Sluka 1998). On the other hand, when hyperexcitability to knee stimulation was established, the responses to pressure onto the ankle were less facilitated by AgaIVA, but on average no inhibition of the responses was observed (Nebe et al. 1997). This may correspond to the finding that in awake rats secondary heat hyperalgesia could not be reversed when AgaIVA was given after induction of inflammation (Sluka 1998).

P-type VDCCs are mainly located at presynaptic sites where they regulate the release of compounds. The pattern of effects of AgaIVA in this study suggests that P-type VDCCs are involved in the spinal nociceptive processing in a complex fashion. First, the responses to mechanical stimulation of the knee before inflammation were not consistently reduced by AgaIVA. Either P-type VDCCs are not functional in this situation or they are involved in the release of both excitatory and inhibitory transmitters (Mintz et al. 1992; Takahashi and Momiyama 1993) such that the net responses to mechanical stimulation are either slightly enhanced, reduced, or unchanged when P-type VDCCs are blocked by AgaIVA. Second, during development of inflammation AgaIVA most likely blocked the additional release of excitatory compounds. Candidates are excitatory amino acids such as glutamate and neuropeptides such as substance P and CGRP because the release of these compounds is enhanced in this model of inflammation (Schaible et al. 1990, 1994; Sluka and Westlund 1992). During this process AgaIVA obviously acted in the neuronal pathways that are involved in the generation of primary and secondary hyperalgesia. Third, when the hyperexcitability is developed, P-type VDCCs seem to be mainly involved in the maintenance of the enhanced responses evoked by stimulation of the inflamed knee, and they seem to be less important in the pathways activated by stimulation of the noninflamed tissues such as the ankle (Nebe et al. 1997; Sluka 1998). Thus the involvement of P-type VDCCs in synaptic responses is more dependent on particular conditions than the involvement of N-type VDCCs. It is not precisely known at this stage how the change in effectiveness of AgaIVA in different phases of inflammation can be explained. At present we do not know which neuronal mechanisms bring P-type VDCCs into play. Furthermore, we do not know in which types of neurons (primary afferent neurons, interneurons) P-type VDCCs are expressed and which transmitters are particularly released by activating presynaptic P-type VDCCs.

As mentioned previously, N-type VDCCs seem to be involved in the synaptic nociceptive processing in a more uniform way than P-type VDCCs. Indeed, N-type VDCCs are activated during synaptic processing of input from the normal and from the inflamed tissue (Diaz and Dickenson 1997; Malmberg and Yaksh 1994; Neugebauer et al. 1996c). Furthermore, ω-conotoxin GVIA, a selective antagonist at N-type VDCCs, reduces nociceptive responses to ~50% (Neugebauer et al. 1996c), thus suggesting that N-type VDCCs play a quantitatively greater role than P-type VDCCs in the synaptic processing in the spinal cord.

In summary, this study showed that P-type VDCCs are involved in the generation of inflammation-evoked hyperexcitability of spinal cord neurons. Furthermore, the results emphasize a role of P-type VDCCs in the maintenance of hyperexcitability once it is established. The blockade of P-type VDCCs may therefore be considered a target for the prevention or reduction of inflammation-evoked pain. However, because of the limited effectiveness of AgaIVA, it is unlikely that the blockade of P-type VDCCs alone will be sufficient to reduce inflammation-evoked hyperalgesia and pain.

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Present address and address for reprint requests: H.-G. Schaible, Institut für Physiologie, Friedrich-Schiller-Universität Jena, Teichgraben 8, D-07740 Jena, Germany.


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