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Construction and properties of a pyrazinamide-selective biosensor using chemoreceptor structures from crayfish

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Abstract

Receptor-based biosensors using structures from fresh-water species (crayfish) are shown to give selective analytical responses to the antitubercular drug pyrazinamide The construction of such biosensors, in general, is greatly facilitated through the use of a video imaging inverted biological microscope during the manipulation and assembly of the delicate structures involved and points the way toward microscale receptors with enhanced analytical properties in the future Characterization of the sensor in terms of selectivity, sensitivity, response time, dose–response relationship and lifetime is reported Advantages of using fresh-water species in constructing neuronal biosensors, e g , extended lifetime and fewer interferences, are also discussed

Keywords Potentiometry, Biosensors, Chemoreceptors, Crayfish, Pyrazinamide

Since the first report of neuronal biosensors based on the use of intact chemoreceptor structures in 1986 [1], analytical dose–response relationships have been demonstrated for numerous natural and synthetic biomolecules [2] Such "receptrode" biosensors have been shown [3] to have extraordinary sensitivities coupled with response times of the order of milliseconds A high degree of selectivity has also been achieved either through the inherent selectivity of native neuronal receptors [4] or, when that is not easily accomplished, through the use of real-time chemometric techniques to extract selective responses from mixed neuronal data [5]

Research on such neuronal biosensors is greatly facilitated through the introduction of color video microscope technology for the visualization and manipulation of neuronal structures under high magnification and the production of permanent visual and data records As described in this paper, the combination of video imaging with the neurophysiological techniques described earlier [6] substantially improves the reliability and convenience of neuronal biosensor construction while extending the range of experimentation to new sources of chemoreceptor structures

All of the work reported to date in this field has been based on the use of various crab species as the source of chemoreceptor structures Crabs are attractive candidates for neurophysiological research because of their wide availability, extensively studied behavioral correlations and the relatively easy exposure of their chemoreceptive areas owing to the size of the neuronal structures involved For analytical purposes, however, crab-based neuronal biosensors are severely limited by the need to maintain a specialized electrolytic medium in all solutions (including the analyte) used and because of technical problems of maintaining viable crab neurons in the laboratory

This paper reports on the first use of neuronal structures from crayfish in biosensor construction and demonstrates their satisfactory analytical behavior in ordinary tap water (or distilled water) as

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the analyte medium It is also shown that such crayfish-based receptrodes offer better thermal and time stability than corresponding crab-based systems and thus yield sensors with improved lifetimes

In addition to such operational advantages, it was also found that the use of crayfish neuronal structures in biosensor construction offers an extension of analytical capabilities to compounds not previously measured In particular, results are presented of a study focused on the sensitive measurement of the important antitubercular drug pyrazinamide with high selectivity over structurally related compounds

Pyridine analogs such as pyrazines occur widely in plants and animal natural products [7,8] Some of these pyrazines have extremely potent aroma and flavouring properties Behavioral experiments have shown that pyridine or its analogs also act as stimuli to the human gustatory and olfactory systems [7,9,10] Pyrazines such as 2,5-dimethyl-3-isopentylpyrazine and 2,6-dimethyl-3-ethyl-, -propyl-, -butyl- and pentylpyrazines function as powerful releasers of alarm behavior for Odontomachus workers and are probably utilized as defensive compounds [8]

The sensory system of the crayfish is provided with a large number of setae Which of these are chemosensory, mechanoreceptors, pressure or temperature receptors remains problematical In some instances, chemoreceptors are closely associated with the mechanoreceptors that cause tactile responses Food detection in crayfish is largely a function of the specialized setae and aesthetascs located in the under side of the outer flagellum of the antennule [11,12] Chemoreceptors are also located in other parts of the crayfish such as antennae, chelae and dactyl of the first and second walking legs and certain mouth parts [11,13,14]

Recent electrophysiological studies of chemoreceptors located on the walking leg of crayfish revealed that four different pyridine analogs can cause significant stimulation [13] The effectiveness of these as chemostimulants decreases in the order pyrazinamide ($K_M = 1\ 5 \times 10^{-6}$) > 3-acetylpyridine ($K_M = 4 \times 10^{-6}$) > nicotinamide ($K_M = 1 \times 10^{-5}$) > pyridine-3-aldoxime ($K_M = 4 \times 10^{-5}$), where K_M is the stimulus concentration at which a half maximum response is elicited Pyrazinamide was the most effective chemostimulant of 79 pyridine analogs tested In this study, chemostimulant potentials of the above four pyridine analogs were tested with the receptor units of crayfish antennule

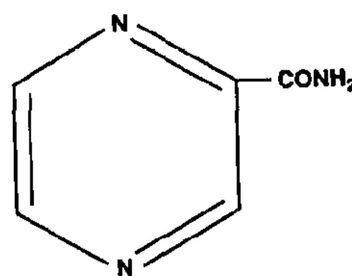

Fig 1 Structure of the antitubercular drug pyrazinamide

Pyrazinamide (Fig 1) has been used in the USA primarily for the retreatment of tuberculosis when the disease is a greater threat than the drug's potential toxicity Doses exceeding 35 mg kg^{-1} per day can cause hepatic and liver damage and a number of fatal cases are on record A convenient and reliable assay procedure for monitoring the mode of action and the dose–response behavior of pyrazinamide has not yet been established The electrophysiological experiments described in this paper demonstrate a new approach to developing novel sensors for the determination of such clinically important drugs

EXPERIMENTAL

Reagents

The pyridine analogs pyrazinamide, niacinamide, 3-acetylpyridine and pyridine-2-aldoxime and other possible stimulants such as adenosine monophosphate, taurine, kainic acid, glutamic acid, norepinephirine, betaine, taurocholic acid, glutamic acid, γ-aminobutyric acid, *cis*-8-dodecyl acetate, menthol, butanol, sucrose and nicotinamide adenine dinucleotide were purchased from Sigma All stock solutions and serially diluted solutions for certain stimulants were prepared in tap water and stored at 5 °C The neuro-bathing solution was prepared in deionized water and had the following composition Na^+ 205, K^+ 5 4, Ca^{2+}

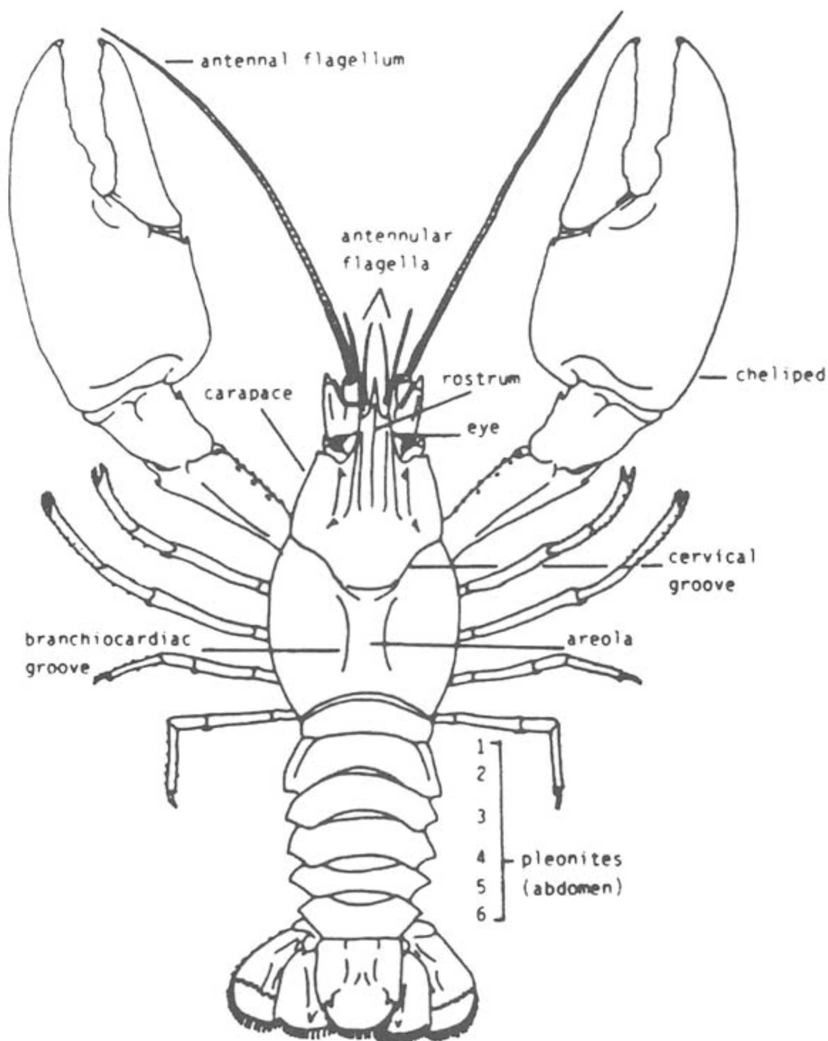

Fig 2 Schematic diagram of a crayfish showing anatomical features

13 5, Mg^{2+} 2 6, Tris malate buffer 10 mmol l^{-1} (pH 7 6)

Crayfish antennules

The crayfish (*Procambarus clarkii*) were obtained from the Manoa stream near the campus and maintained in a fresh-water tank for later use An anatomical diagram of a crayfish is shown in Fig 2 In this study, an antennular section of the crayfish was used as the chemosensitive unit Each crayfish has four antennules which could be used effectively for different experiments Figure 3 shows a close-up view of the distal end of a crayfish antennule as seen under the phase contrast microscope (75 ×) The actual length of an antennule can vary with the age of the species and is of the order of 1–2 cm Different types of receptors such as chemosensory receptors, mechanoreceptors, and pressure or temperature receptors are located in the aesthetascs (the fine hair-like structures) of the antennules The individual aesthetascs are of micrometer size and require special equipment for visualization and manipulation Figure 4 shows a single aesthetasc, under high magnification, photographed from the video screen of the microscope monitoring system This delicate structure represents the ultimate size limit and goal for chemoreceptor structure-based sensors

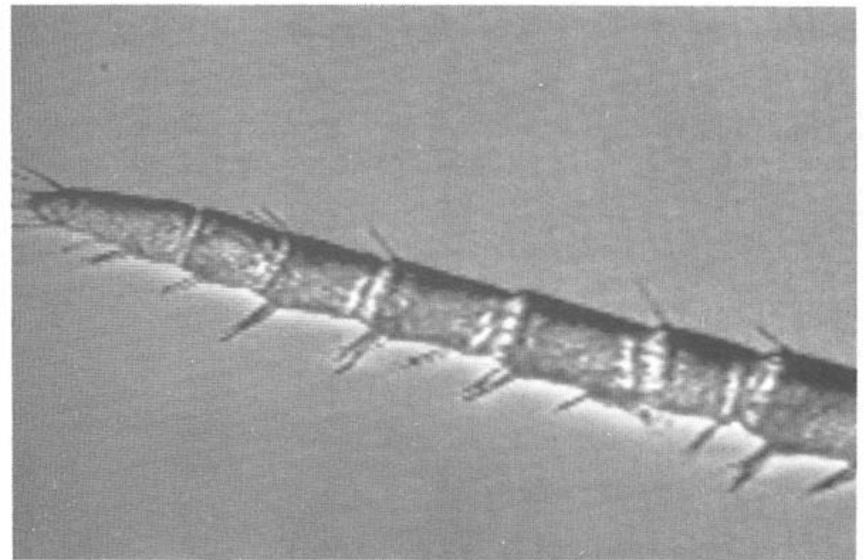

Fig 3 Video image of the distal end of a crayfish antennule (magnification 75 ×)

Apparatus

A schematic diagram of the apparatus used is shown in Fig 5 The Plexiglas cell was designed to hold the antennules in place on the mechanical stage of the Bio-star trinocular dissecting microscope The cell mainly consists of a circular chamber attached to a carrier stream line The

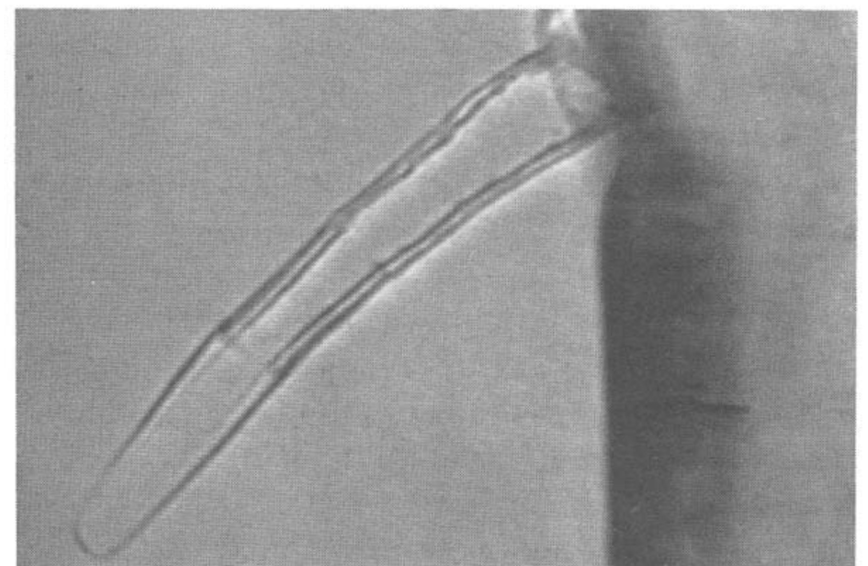

Fig 4 Fine structure of an individual aesthetasc under high magnification (850 ×)

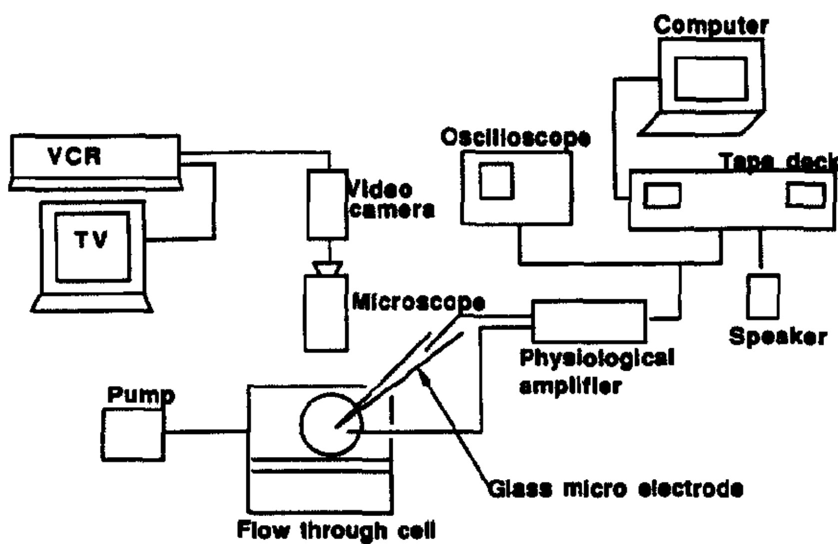

Fig 5 Schematic diagram of the experimental set-up

neurobathing chamber (circular bath) and the carrier stream line were connected to each other by a small hole Thus, crayfish antennules can be mounted in such a way that the nerve fibers are exposed to the neurobathing solution and the chemosensitive portions to the fresh-water carrier stream for testing possible stimulants Reference and ground wires were inserted into the neurobathing chamber

A glass micropipette containing an Ag/AgCl element was also positioned in this chamber, with a micromanipulator, to measure the action potentials of different nerve cells A small amount of neurobathing solution was drawn into the micropipette with a suction syringe in order to create a conducting medium between the Ag/AgCl element and the nerve cells Once a good seal had been made between the tip of the micropipet and the nerve cells, action potentials can be recorded A solution carrier stream was pumped through the Plexiglas cell using a Rabitplus digital peristaltic pump A four-port, two-way valve with a sample injection loop was used to apply a constant concentration of stimulus solution to the chemoreceptors for a period of 10 s The indicator electrode potential with respect to ground and reference was amplified 10, 100 or 1000 times, as appropriate, using a Grass Instruments neurophysiological preamplifier Output of the amplifier was monitored on Tekronix 564 B storage oscilloscope and also stored on an audio tape using a TEAC v-385 stereo cassette recorder A microphone enabled an oral account of the experiments to be stored in one channel of the stereo tape while the data were stored on the other channel Action potential data analyses were done using an IBM Model (c) DTK 386 BIOS Ver 4 25 personal computer

The Bio-star Model 1820 inverted biological microscope used for this experiment was capable of magnifying the objects in a range from 40 × to 450 × Use of a phase contrast unit was particularly important to visualize clearly the depth of the antennule and the aesthetascs The video unit coupled to the microscope system consisted of a video camera, a color monitor and a video recorder A Hitachi vk-c150 color camera featuring 340 lines horizontal resolution, 15 734 and 59 94 kHz horizontal and vertical scanning frequency, respectively, 46 dB signal-to-noise ratio and 3 579545 color carrier frequency is mounted on the trinocular tube of the microscope The signal output of the video camera was displayed on the Sony color video monitor (Model PVM-1380) with a resolving power of 250 TV lines The video unit attached to the microscope produced highly magnified images ranging from 150 × to 1700 × All video images were stored on a video tape, using a JVC video cassette recorder (Model HR-D660U), for later use Color pictures of the fine structure of an antennule were obtained by photographing the video screen using a 35 mm camera (Chinon CM-5)

Procedure

On dissecting the upper part of the crayfish antennules (2–3 mm), nerve fibers can be visualized through the microscope The dissected antennule was carefully mounted on the flow-through cell so that a considerable length of the bottom part of the antennule, which contains chemosensory hairs (aesthetascs) remains in the carrier stream and the exposed nerve fibers are placed in the circular chamber Neurobathing solution was added immediately, tap water was also pumped continuously as a carrier stream to prevent possible damage to the exposed nerve cells and the remainder of the antennule from dehydration Ground and reference wire electrodes were connected to the physiological amplifier The tip of the micropipet electrode was posi-

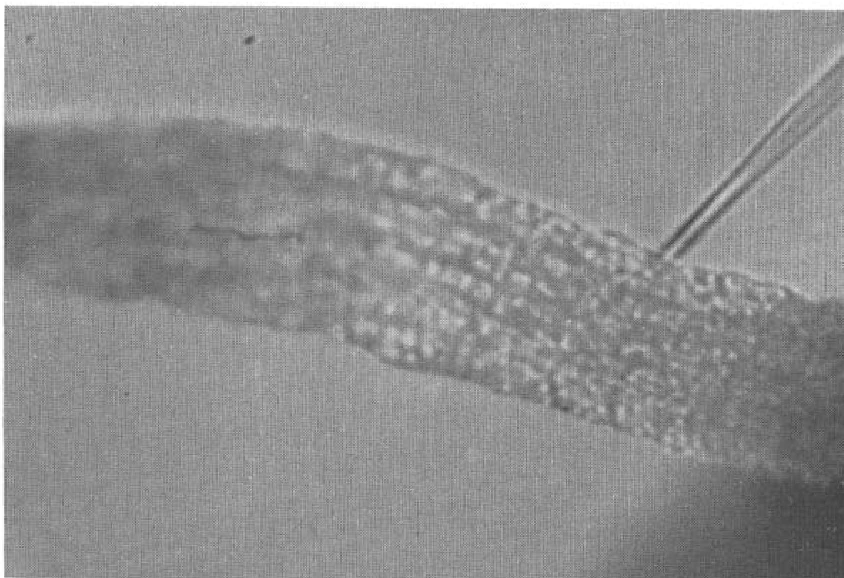

Fig 6 Video image of the nerve fiber with the glass capillary electrode in place (magnification 375×)

tioned on the exposed nerve fibers until action potential spikes were observed and heard on the audio speaker The video monitoring system is particularly helpful in viewing the contact position and positioning the contact electrode as shown in Fig 6 Once a significant action potential is observed, various stimulants can be injected into the carrier stream using the sample injection valve The volume of the sample loop was 25 μl

Serially diluted analytes were injected for analytes which showed significant stimulation to construct dose–response curves The frequency of baseline action potentials (no stimulant in the carrier stream) was also recorded and subtracted as a "blank" from the signals produced by the chemostimulants Segments of action potentials corresponding to background response and stimulant response at the maximum were selected and digitized at a rate of 50 kHz for 5 12 s using the "einsight" chemometric data analysis program Frequency of firing was calculated from the histogram plots which show the number of potential spikes that fall into different amplitude ranges (in mV) Actual frequency response (stimulant response − background) was plotted against the stimulant concentration or log(stimulant concentration) where appropriate to obtain dose–response plots The selectivity of the sensor was tested by injecting some structurally related compounds and also other possible stimulants such as essential amino acids, excitatory amino acids, neurotransmitters, agonists and antagonists The lifetime of the sensor was also studied by injecting primary stimulant (1 mM pyrazinamide) at 1-h intervals until no response could be seen

RESULTS AND DISCUSSION

The molecular recognition units involved in the construction of intact chemoreceptor-based sensors are extremely small and can only be visualized using a high-power (magnification) microscope However, manipulation has to be done by viewing such small units through the eye-pieces of a microscope This method is not convenient (in some instances it is impossible) when the fine structure of receptors cannot be visualized clearly This difficulty was significantly eliminated by using the modern video image technology described in this paper Video camera/monitor units attached to the microscope produced excellent color pictures of fine structures while magnifying the objects beyond the limits of the microscope As seen here, total magnification up to 1700 × could be achieved using the video unit compared with the 450 × highest possible magnification obtained using the microscope itself

Higher magnification is essential in constructing neuronal biosensors using fine structures of various species Figure 4 illustrates the video image of an aesthetasc (850 ×) on the crayfish antennule Typical length and diameter of the aesthetasc were 102 and 12 μm, respectively, as measured from the video image As described earlier, different types of receptors are located on the nerve cells inside these aesthetascs Binding molecules can penetrate into the receptor cells and cause depolarization of potential gradients across the cells Equilibrium is re-established very quickly after returning to the resting potential Potential spikes are generated from this rapid ion channel equilibrium process and the signal is propagated along the nerve cells to the brain In early work, such action potentials were recorded from a remote end of the nerve fibers using a glass capillary electrode (Fig 6) The optimum contact position of the electrode with the nerve cells needs to be determined in order to obtain selective responses from different stimulants, this

process is greatly facilitated using the manipulation/video imaging techniques described here and permits a more systematic approach to receptor identification

In the future, a further benefit may be expected from the video-assisted micromanipulation techniques proposed here Specifically, it may become possible to connect the contact electrode directly to the stem of an individual aesthetasc (see Fig 4) to prepare micro-scale receptrodes Such an arrangement would also result in stronger and cleaner signals, perhaps with even greater selectivity, than the present sensors which rely on electrical contact at the efferent neuronal fiber (Fig 6) Realization of this goal requires the use of contact micropipets having diameters in the 1–2-μm range, some technical problems associated with the use of such fragile probes are under investigation

This paper also introduces the use of fresh-water species, e g , crayfish, as the source of chemoreceptor structures The use of crayfish significantly reduces the interference effects introduced by the need for sea-water matrices and simplifies the experimental protocol In nature, sea-water crabs are very sensitive to external variations such as temperature and salinity changes in their environment Thus, sensors made using antennular sections of crabs also show the same behavior In some instances, these sensors provide false-positive results irrespective of the analyte tested because of such external variations As crayfish are fresh-water species, tap water or deionized water could be used effectively for both carrier stream and analyte media for the development of crayfish neuronal sensors Chemoresponse data obtained from these sensors provide more reliable information owing to less interference from the background The excellent thermal stability of crayfish receptors also provides some advantages Temperature differences between the analyte and the carrier solution have no significant effect on the action potential measurements As reported earlier [15], the activity of amino acid-sensitive receptors in the crayfish walking leg was not affected within a broad temperature range from 0 to 26 °C Therefore, action potential measurements were carried out at the room temperature (22 °C) without special temperature control of the flow-through cell

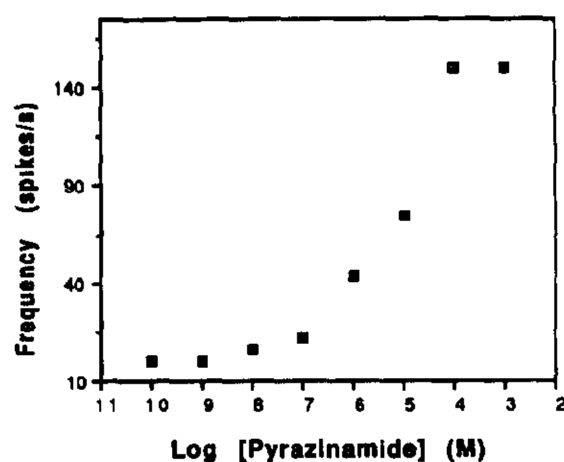

Fig 7 Multi-unit dose–response curve of the sensor to pyrazinamide

Pyrazinamide represents a potential chemostimulant for the receptors in crayfish antennules as revealed during the screening test of the sensor with structurally related pyridine analogs and other reported chemostimulants Repeated chemoresponses from several crayfish antennules confirmed the presence of receptor cells sensitive to pyrazinamide

Multi-unit dose–response

The dose–response curve obtained from serially diluted pyrazinamide solutions is shown in Fig 7 This particular data set corresponds to multi-unit action potential responses Multi-unit data consist of potential spikes of varying amplitude Extracting the individual units responding to a chemostimulant is complicated owing to the complex nature of the response pattern In some instances, multi-unit responses can be seen when chemoreceptors are closely associated with the mechanoreceptors causing both tactile and chemoresponses Two or more action potential units having different amplitudes may also respond to a particular stimulant simultaneously, creating such multi-unit response patterns Window discriminators have been used earlier to distinguish and identify selectively action potential spikes obtained from various chemostimulants As reported earlier [3], this method was reliable only for the analysis of single-unit response data In this study, multi-unit responses were analysed using a chemometric data analysis

program Histogram plots showing the number of potential spikes falling into different amplitude ranges (mV) were obtained for each of the serially diluted analytes The response frequencies in each bin of the histogram plots were compared to extract the best data set which shows a linear relationship with the analyte concentration A detailed description of the data analysis techniques is reported elsewhere [16] A broader concentration range of the stimulant was initially used to determine the dose–response behavior Figure 7 indicates an increase in response from 10^{-7} to 10^{-4} M analyte concentration The lack of change in frequency response above 10^{-4} M pyrazinamide may be due to the saturation of receptors causing adaptation The frequency response shows a linear relationship with the concentration of pyrazinamide from 10^{-5} to 10^{-4} M This was investigated further by injecting serially diluted pyrazinamide solutions within a narrow concentration range

Single-unit response patterns

Single-unit response of the sensor to 3×10^{-5} M pyrazinamide, as monitored on the oscilloscope, is shown in Fig 8a together with the background activity (Fig 8b) Actual potential responses were amplified by a factor of 1000 using a low-noice differential a c preamplifier

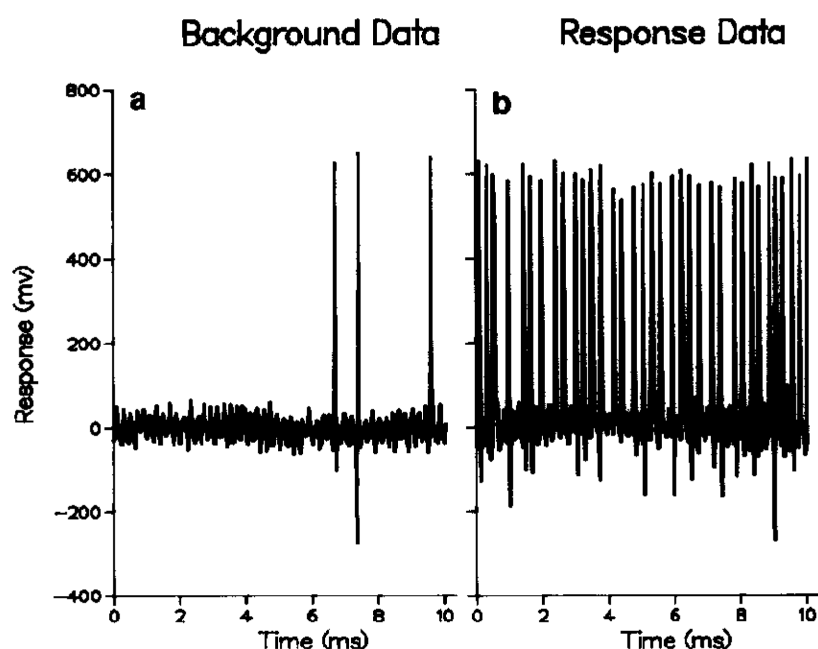

Fig 8 (a) Record of single-unit response of the sensor to 3×10^{-5} M pyrazinamide, (b) background response of the sensor prior to the injection of analyte

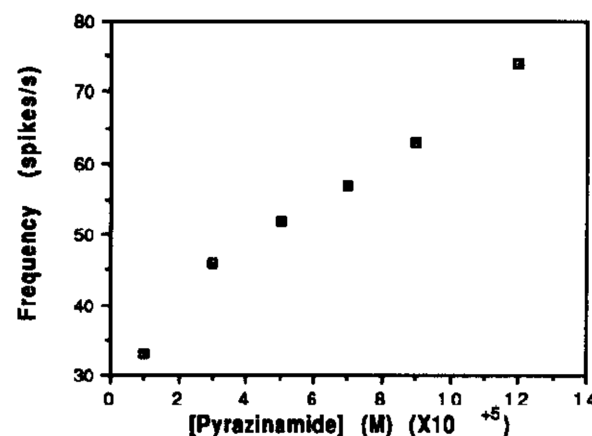

Fig 9 Single-unit dose–response curve of the sensor to pyrazinamide

Increased frequency of potential spikes is a clear indication of chemostimulation by pyrazinamide The approximately equal magnitude of the potential spikes indicates a typical single-unit response pattern and the time scale of these plots also shows the response time of the sensor to be of the order of a few milliseconds As reported previously [1,3–5], such short response times are typical for intact chemoreceptor-based sensors owing to rapid ion transport on substrate binding to the receptor site Acetylcholine receptors have been used extensively as a model to explain the ion channel opening mechanism

Single-unit dose–response

Figure 9 represents a single-unit dose–response curve obtained with pyrazinamide Single-unit response data are easy to analyse and also very reliable in terms of quantitative measurements As the magnitudes of the potential spikes are almost the same, frequency of firing can be easily calculated In this instance, only one bin of the histogram plots indicates the response frequency due to the potential spikes of same amplitude The slope of the dose–response curve indicates a linear relationship with the analyte concentration, such a linear range is useful for determining an unknown concentration of the analyte Recent experiments with single unit data from several crayfish antennules produced almost identical maximum frequency responses (spikes per second), demonstrating good reproducibility of the neuronal sensor The maximum response frequency of 42 ± 1 spikes s^{-1} was obtained with

three antennules from different crayfish for 1 mM concentrations of analyte

Selectivity measurements

One remarkable feature of intact chemoreceptor-based sensors is their high degree of selectivity Such selectivity is also demonstrated in this study Over fifteen different analytes, including essential amino acids, excitatory amino acids, neurotransmitters, hormones, alcohols, sugars, agonists, antagonists and structurally related compounds, were tested as possible stimulants together with the primary stimulant pyrazinamide Only the primary analyte yielded a response, indicating the unique binding properties of the chemoreceptors

Lifetime

The lifetime of the crayfish sensors was much better than those of corresponding crab antennular-based sensors Typical lifetimes were of the order of 8–10 h depending on the concentration of the analyte injected In some instances, injection of highly concentrated analyte (> 1 mM) diminished the response within 2–3 h, perhaps because of nerve cell damage Excised antennule can be kept viable for more than 2 days in saline media at 15 °C Detailed investigations on extending the lifetime of these sensors will be reported in the future Extension of lifetimes is a critical factor in the effort to develop reusable sensors

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REFERENCES

1 S L Belli and G A Rechnitz, Anal Lett , 19 (1986) 403
2 S L Belli and G A Rechnitz, Fresenius' Z Anal Chem , 331 (1988) 439
3 R M Buch and G A Rechnitz, Anal Lett , 22 (1989) 2685
4 R M Buch and G A Rechnitz, Biosensors, 4 (1989) 215
5 R M Buch, T Q Barker and G A Rechnitz, Anal Chim Acta, 243 (1991) 157
6 R M Buch and G A Rechnitz, Anal Chem , 61 (1989) 533A
7 H J Takken, L M van der Linde, M Boelens and J M van Dort, J Agric Food Chem , 23 (1975) 638
8 J W Wheeler and M S Blum, Science, 182 (1973) 501
9 R M Seifert, R G Buttery, D G Guadagini and J G Harris, J Agric Food Chem , 20 (1972) 135
10 H D Belitz, W Chen, H Jugel, R Treleano and H Wiser, in J C Boudreau (Ed), Food Taste Chemistry (ACS Symposium Series, No 115), American Chemical Society, Washington, DC, 1979, pp 93–131
11 E S Hodgson, Biol Bull (Woods'Hole, Mass), 115 (1958) 114
12 W S Thomas, J Zool , 160 (1970) 91
13 H Hatt and I Schmiedel-Jakob, J Comp Physiol A, 154 (1984) 855
14 C Ameyaw-Akumfic, Crustaceana, 33 (1977) 259
15 H Hatt, J Comp Physiol , 152 (1983) 405
16 T Q Barker, R M Buch and G A Rechnitz, Biotechnol Progr , 6 (1990) 498