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Development and validation of a cellular biosensor detecting pesticide residues in tomatoes

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ABSTRACT

Two of the most important categories of pesticides used in agricultural practice are organophosphates and dithiocarbamates. Their extensive and inappropriate use has rendered their reliable monitoring at trace levels more and more necessary. This study presents the construction of a rapid and sensitive cellular biosensor test based on the measurement of changes of the cell membrane potential of immobilized cells, according to the working principle of the Bioelectric Recognition Assay (BERA). The cells were immobilized by entrapment in a sodium alginate bead and directly applied in different pesticide dilutions and agricultural samples. The pesticides used were the organophosphate insecticide diazinon and the dithiocarbamate fungicide propineb. Two different cell types, N2a (neuroblastoma) and Vero (fibroblast) were used as the biosensory elements in order to investigate their differential response against the pesticides. In this way, we hoped to increase the selectivity of the assay. Based on the observed patterns of response, we demonstrate that the sensor can be used for the qualitative and, in some concentrations, quantitative detection of the pesticides with a high degree of reproducibility. The lowest detected concentration was 3 nM. Finally, for the investigation of the effects of different pesticides on the accumulation of cytosolic Ca^{2+}, we conducted a fluorescent assay on N2a cells treated with tomato sample extracts, which were replicates of the E.U. proficiency test sample. The tomato samples were either organically grown or contained 14 different pesticides. The experimental results showed a higher increase of the intracellular Ca^{2+} concentration in cells treated with non-organic samples compared to the cells treated with organic samples.

1. Introduction

During the last two decades there have been growing societal concerns over issues related to public health, environmental quality, and food safety. One of the major controversies inciting these concerns involves the production and consumption of fresh fruits and vegetables [1]. It is well known that the level of risk is closely related to the daily intake of chemically contaminated food and that for certain products which are frequently consumed the risk is higher than others [2]. Tomato (*Lycopersicon esculentum*) is one of the most widely grown vegetables in the world and a basic component of the Mediterranean diet and is used almost daily in several European countries, raw, home-cooked or processed as a canned product, juice or paste [3].

In environmental pollution monitoring, it is becoming a general opinion that chemical analysis by itself does not provide sufficient information to assess the ecological risk of polluted waters and wastewaters [4]. Although conventional techniques such as HPLC/MS and GC/MS give satisfactory analytical results for pesticide determination, new assays and sensors for cheaper and faster on-site analysis are being developed.

Biosensors are being developed for different applications, including environmental and bioprocess control, quality control of food, agriculture, military, and, particularly, medical applications. In fact, most of the commercially available biosensor systems are applied in the clinical and pharmaceutical markets. Accordingly, most research and development has been devoted to this area. In the food industry, the detection of contaminants, verification of product content, monitoring of raw materials conversion and product freshness [5] are areas of potential biosensor application.

Based on the inhibition of acetylcholinesterase (AChE) and choline oxidase, many biosensors have been developed for the detection of organophosphorous and carbamate pesticides such as those described by Andres and Narayanaswamy [6], Choi et

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al. [7], Andreou and Clonis [8] and Mavrikou et al. [9]. The biological effects of organophosphorous (OPs) insecticides such as diazinon are exerted by reversible or irreversible inhibition of acetylcholinesterase (AChE), which results in decreased catalysed hydrolysis and excessive accumulation of extracellular acetylcholine (ACh). The ensuing hyperactivation of ACh receptors results in various toxic effects including hypersecretions, convulsions, respiratory distress, coma, and ultimately death [10,11].

Propineb is a fungicide with the propylene-bis-dithiocarbamate structure. It is widely used in fruit and vegetable horticulture. Dithiocarbamates (DTCs) form lipophilic complexes with di- and trivalent metallic cations, bonding through the sulfur atoms. In the case of propineb, a zinc ion is chelated. It was shown that Zn^{2+} concentration is dose-dependently accumulated in liver and kidney tissue [12]. In addition, DTCs-induced neurobehavioral and extrapyramidal symptoms have been related to the impairment of central neurotransmitter release mechanism; alteration of dopamine [13–15], noradrenaline, and acetylcholine (ACh) [16] levels were mainly observed in rat and mouse brain areas after DTCs administration.

Interactions of mammalian cells with pesticides are frequently associated with changes in cell membrane functions: in the case of neuronal cells these changes are associated with altered responses to neurotransmitters, such as ACh. The presence of pesticidal compounds is directly associated with changes of the immobilized cell membrane potential. Even changes associated with non-membrane effects, such as the cytoplasmic induction of apoptotic pathways will frequently involve changes in calcium ion (Ca^{2+}) concentrations that could be eventually translated in changes of the membrane potential [17]. In this way, a pesticide assay could be based on electrically active cells, interfaced with microelectrodes which allow the capture of extracellular spikes or impedance changes associated with cellular response against the pesticide under detection. An example is the Bioelectric Recognition Assay (BERA) which has been originally developed for the detection of viruses on the basis of their specific interaction with appropriately immobilized, mammalian cells and the measurement of the change of the electric potential that is caused by the aforementioned interaction [18,19]. It has been recently demonstrated that the assay could be used for the detection of the organophosphate pesticide chlorpyriphos and the carbamate carbaryl in a concentration-dependent pattern [9]. According to the working principle of the method, the presence of the pesticides is detected by the degree of inhibition of cellular AChE, which is inversely associated with ACh concentration. Inhibition of AChE lead in increased excitatory ACh transmission and depolarization of the cell membrane, which was measured as a change of the sensor's potential, due to changes in the concentration of electrolytes in the immediate vicinity of the working electrode. This methodological approach displays many advantages over other biosensor techniques, such as high speed (assay time = 3 min), sensitivity (1 ppt to 1 ppb, depending on the pesticide), reproducibility and low cost. However, the selectivity of the assay is questionable, because a given cell type can react in roughly the same manner against a large number of different pesticides (a common problem in cell-based toxicity assays).

One method to increase the selectivity of the assay could be the use of different cell types having a different response pattern to the assayed pesticides. Therefore, in the present study we report the development of a cellular biosensor utilizing the BERA working principle and based on neuroblastoma N2a or Vero cells, two cell types quite different in their physiology. The novel sensor was tested against standard solutions of the organophosphate insecticide diazinon and the dithiocarbamate fungicide propineb and validated against tomato samples containing a mixture of pesticides.

2. Experimental

2.1. Materials

Mouse neuroblastoma (N2a) and monkey African green kidney (Vero) cell cultures were originally provided from LGC promochem (UK). Diazinon (diethoxy-[(2-isopropyl-6-methyl-4-pyrimidinyl)oxy]-thioxophosphorane; CAS [333–41–5]; Mw = 304.35; Sigma–Aldrich, USA) was used as a standard organophosphate insecticide and propineb ([[2-[(dithiocarboxy) amino]-1-methylethyl]carbamodithioato]](2-)-k*S*,k*S*′] zinc; CAS [235–134–0]; Mw = 289.80; Bayer Cropscience, Germany) as a standard dithiocarbamate fungicide. Pesticide mixtures which contained 10 μM of each pesticide, were prepared daily in acetone solution. All other reagents were purchased from Fluka (Switzerland).

2.2. Cell culture and sensor fabrication from N2a and Vero cells

Cells were cultured in Dulbecco's medium with 10% heat-inactivated fetal calf serum (FCS), 10% antibiotics (streptomycin) and 10% L-glutamine. After cell detachment from the culture vessel by adding trypsine/EDTA for 10 min at 37 °C and cell concentration by centrifugation (6 min, 1200 rpm, 25 °C), 1 ml of cells (at a density of 2.5×10^6/ml) were mixed with 2 ml of 4% (w/v) sodium alginate solution and then the mixture was added drop wise, by means of a 22G syringe, in 0.8 M $CaCl_2$. Each of the resulting calcium alginate beads had an approximate diameter of 2 mm and contained approximately 5×10^4 cells.

The sensors were storable at room temperature in culture medium, under normal atmospheric conditions (i.e. non-CO_2 enriched) for at least three weeks without any loss of performance.

2.3. Sample preparation

Thirteen blended tomato samples were analysed. Five of them were replicates of a control sample derived from strictly organically grown tomatoes, free of pesticide residues while the rest of them were replicates of the E.U. proficiency test sample containing a mixture of fourteen pesticides at concentrations ranging from 0.05 to 3.955 mg/kg (Table 1).

Standard aqueous solutions of either diazinon or propineb were prepared in the range of concentrations 0.003, 0.016, 0.03, 0.16, 0.33, 1.65 and 3.30 μM in water. The concentrations 1.65 and 3.30 μM of the pesticides had to be dissolved in a small amount of ethanol and diluted with water (5%, v/v) because of their insolubility in water.

2.4. Assay principle

According to the working principle of the method, the presence of organophosphate compounds is detected by the degree of inhibition of cellular AChE, which is inversely associated with ACh concentration. ACh is an excitatory neurotransmitter [20], the activity of which is regulated by AChE. Therefore, inhibition of AChE can lead in increased excitatory ACh transmission, which can be measured by the depolarization of the cell membrane. In other words, inhibition of AChE by pesticide residues in the sample will result to excessive stimulation of N2a cells by ACh, which will further lead to membrane depolarization above a pre-determined threshold. The presence of DTCs is also detected by the increase of ACh concentration: it has been previously reported that propineb (0.001–100 nM) dose-dependently increases ACh release from PC12 differentiated by NGF [21]. In addition, previous data obtained from Marinovich et al. [22] indicated a possible involvement of nicotinic

Table 1
Fourteen pesticides contained in E.U. proficiency test tomato samples.

Acrinathrin0.284 mg/kg	Azoxystrobin 0.225 mg/kg	Bromopropylate 0.490 mg/kg	Chlorothalonil 1.626 mg/kg	Diazinon 3.955 mg/kg
Dimethoate0.132 mg/kg	Endosulfan 0.344 mg/kg	Imazalil 0.167 mg/kg	Imidacloprid 0.232 mg/kg	Dithiocarbamates 0.810 (as CS_2)
Metalaxyl 0.050 mg/kg	Oxydemeton methyl 0.199 mg/kg	Procymidone 0.412 mg/kg	Thiabendazole 0.314 mg/kg	

receptors present on nerve terminals in propineb-induced ACh release.

Correspondingly Vero cells were used as a non-responsive biosensory element for the case of organophosphates. On the other hand propineb may exert a high sensitivity response in kidney-derived Vero cells due to its Zn-containing molecule: zinc concentration varies widely in different tissues, but Zn is known to accumulate in two particular organs, namely the liver and kidney, where it may cause biochemical and histopathological changes [23,24]. According to Davies et al. [25] and Houtani et al. [26] zinc-activated ion channel (ZAC) is a mammalian protein, expressed in prostate, thyroid, trachea, lung, brain (adult and fetal), spinal cord, skeletal muscle, heart, placenta, pancreas, liver, kidney and stomach. The L2 sequence of ZAC gene is conserved among species including human, chimpanzee, dog, cow, and opossum [26]. Membrane depolarization events and associated electrolyte influx/efflux will reflect themselves on the sensor's response as a change of the sensor's potential, due to changes in the concentration of electrolytes in the immediate vicinity of the working electrode.

2.5. Assay procedure

Each cell-bearing bead (cell sensor) was manually connected to a working electrode (the electrode was inserted through the entire length of the sensor without extruding from the opposite end) made from pure silver, electrochemically coated with an Ag/AgCl layer and having a diameter of 0.75 mm. The distance between working and reference electrode was 3 cm. Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for recording the signal and data processing was InstaCal (Measurement Computing).

For each assay, the sensor system, comprising of the bead attached to the working electrode and a reference electrode, was immersed into each sample solution (200 μl). The response of each sensor was estimated by recording the average change of the sensor potential for a period of 180 s after sample application.

2.6. Fluorescence assays of pesticide–cell interaction

Changes in cytoplasmic Ca^{2+} concentration in N2a cells were measured microscopically, after the addition of pesticide-free tomato samples and tomato samples containing 14 different pesticides were monitored by the uptake of the acetomethyl ester of Fluo-3 [27]. After application of 5 μl of the dye, the fluorescence of the specimens was recorded for 5 min at 10 s intervals. Slides with stained cells were mounted on a Zeiss Axiolab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. A digital camera (SONY S75 digital still camera) was attached to the microscope with adjustable BP-546/FT-580 excitation filter/chromatic beam splitter combinations. In order to control photobleaching, we kept specimen exposure times at a minimum level.

In addition, a fluorescence assay was conducted spectrophotometrically. Cells were plated on special optics 96-well plates (black, clear-bottom; Greiner) coated with poly-L-lysine (100 μg/ml) at a density of 2×10^4 cells per well. After 3 days in culture, Fluo-3 AM (10 μM) was added to the cell suspension of each well, which was subsequently incubated for 60 min at 37 °C. The cells were then washed two times with sterile deionized water. The cells were incubated with either tomato samples or with K^+ (70 mM) in a standard medium (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 20 mm HEPES-Na (final pH 7.4)), which was used for loading and for the subsequent fluorescence measurements. The fluorescence intensity of Fluo-3 was quantified by a fluorescence spectrophotometer (Molecular Devices Spectramax M2e microplate reader), with a single excitation wavelength set at 492 nm and an emission wavelength monitored at 520 nm. All data points were done in triplicates, and experiments were repeated at least three times with different batches of N2a cells.

2.7. Data analysis and experimental design

Both biosensor and conventional sample analysis were conducted according to a double-blind protocol. Experiments were set up in a completely randomized design and each experiment was repeated four times. Results were assessed by a standard analysis of variance. In each application, a set of five biosensors was tested against each individual sample. Data means among different days were compared using Duncan's multiple range test (with significance at $p < 0.05$).

3. Results

3.1. Response of the sensor to standard pesticide solutions

The results of the assay of standard propineb solutions at different concentrations with neuroblastoma and Vero biosensors are shown in Fig. 1A, while the results of assaying standard diazinon solutions with neuroblastoma and Vero biosensors are shown in Fig. 1B. In the case of Vero cells, when no pesticide was present in the sample (control sample), a constant sensor response of -70 ± 5 mV was observed. The sensors responded to propineb by considerable positive increase of the sensor's potential (Fig. 1A). This response was concentration-depended in the range of 0.03–0.33 μM ($r^2 = 0.9816$, $y = 7.788x - 37.758$), so that both the qualitative and the quantitative detection of the pesticide was possible, at least at concentrations lower or close to either 0.33 μM (Vero cells) or 1.65 μM (N2a cells). The biosensor based on N2a cells gave also a concentration-depended response in the range of 0–0.16 μM ($r^2 = 0.9527$, $y = 15.5x - 68.386$).

A similar, concentration-dependent pattern ($r^2 = 0.9046$, $y = 6.4788x - 59.268$) was observed in the N2a sensor's response against diazinon (Fig. 1B), in the range of 0–0.33 μM. In contrast, the Vero cell biosensor's response did not follow a concentration-depended pattern.

Depending on the concentration of each pesticide, representative recovery rates ranged from 51 to 93% for diazinon and from 52 to 122% for propineb (Table 2).

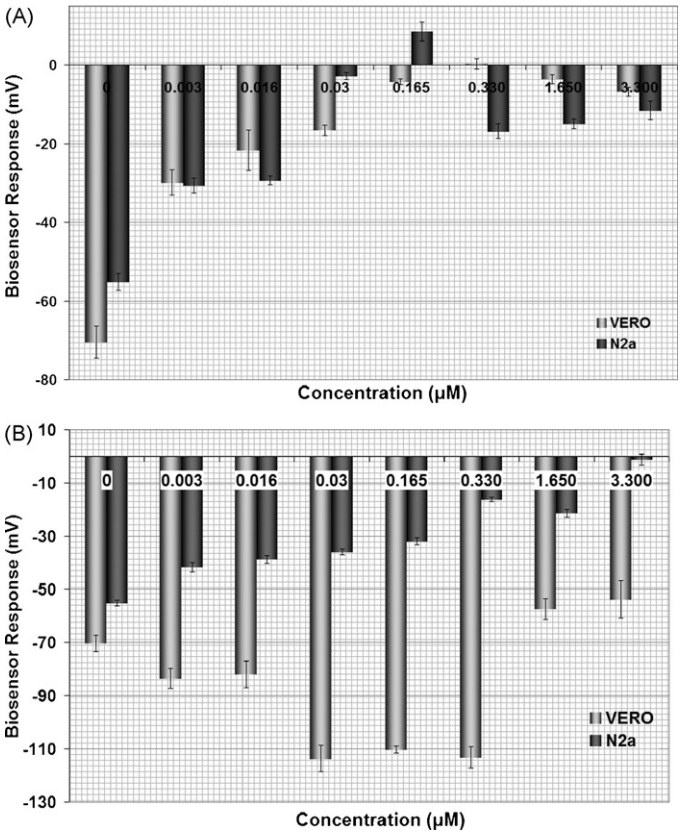

Fig. 1. Biosensor response against standard solutions containing propineb (A) or diazinon (B) at various concentrations. Sensor response is expressed as a change in the membrane potential of immobilized cells. ($n = 15$ replications (different sensors) for each sample and error bars represent standard errors of the average value of all replications with each sample.) The white columns represent Vero cells and the gray columns N2a cells. With each different colour, columns marked with different letters indicate statistically different values ($p < 0.01$).

3.2. Residue profile by conventional analysis of tomato samples

Eight of the thirteen tomato samples contained a mixture of fourteen pesticides which are presented in Table 1, while the biosensor responses for the thirteen tomato samples are presented in the *y* axis of Fig. 2. The white columns represent organic tomato samples and the gray columns represent tomato samples containing the fourteen pesticides in various concentrations. Pesticide residues in tomato samples caused a considerable cell membrane depolarization on neuroblastoma cells immobilized in the sensor, as indicated by the decrease of the negative sensor potential, which was clearly distinguishable from the sensor's response against pesticide-free control samples. There were no false-negative measurements.

Differences in responses between replicates of the control sample may be due to enhanced matrix effect of the tomato samples on the neuroblastoma cells immobilized in the sensor. Tomato is rich in carotenoids, such as lycopene. It has been previously shown that carotenoids and their oxidation products promote gap

Table 2
Recovery rates for diazinon and propineb with the novel cell biosensor assay.

Pesticide	Concentration (μM)	Recovery rate (%)
Diazinon	0.01	51.01
	0.05	92.81
Propineb	0.01	121.93
	0.05	51.62

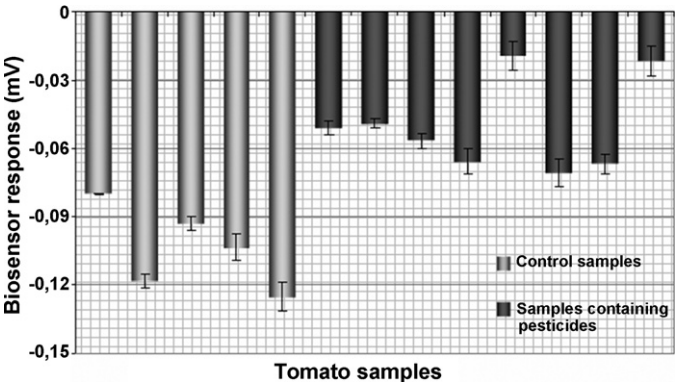

Fig. 2. Biosensor response against thirteen different tomato samples. The sensor was based on neuroblasoma N2a cells. The white columns represent pesticide free tomato samples and the gray columns represent tomato samples containing fourteen pesticides in various concentrations. Sensor response is expressed as a change in the membrane potential of immobilized cells. ($n = 15$ replications for each samples and error bars represent standard errors of the average value of all replications with each sample.)

junctional communication [28]. It is, therefore, quite possible, that differences in the oxidation rate of lycopene and other carotenoids between individual samples resulted in the observed differences in responses between replicates of the control sample.

3.3. Fluorescence assays on calcium uptake by N2a cells after treatment with various tomato samples

Neuroblastoma cells responded to the presence of tomato samples containing 14 different pesticides in various concentrations, by a rapid and very considerable increase of their intracellular Ca^{2+} concentration (Fig. 3), compared to cells treated with pesticide free tomato samples. The spectrophotometric assay gave similar results in the fluorescence intensity (Fig. 4). The pesticide free samples indicated a significant difference compared with the samples containing pesticides. Cells incubated with a Krebs HEPES buffer containing a high concentration of K^+ (70 mM) were used as a positive control, while cells incubated with Krebs HEPES buffer were used as a control. The purpose of the microscopy fluorescence assay was to investigate the participation of Ca^{2+} traffic through the cell membrane as a possible mechanism underlying the observed changes in cell membrane potential, as measured with the biosensor. This measurement was not meant to include a complete fluorescence-based assay for pesticide detection, therefore different pesticide concentrations were not tested.

4. Discussion

Cell-based biosensors constitute a promising field that has numerous applications ranging from pharmaceutical screening to environmental monitoring. Cells provide an array of naturally evolved receptors and pathways that can respond to an analyte in a physiologically relevant manner. Enzymes, receptors, channels, and other signalling proteins that may be targets of an analyte are maintained and, as necessary, regenerated by the molecular machinery present in cells [29].

The results of the present study clearly demonstrate that detection of different pesticide substances with BERA, was primarily based on the specificity of the cell line used in the biosensor. The biosensor presented in this study belongs to a new category of pesticide residue detectors based on mammalian cell lines. Previous studies report the use of genetically modified bacteria for the detection of toxic and especially mutagenic compounds [30]. Even though the biosensor development is at an early stage the biosen-

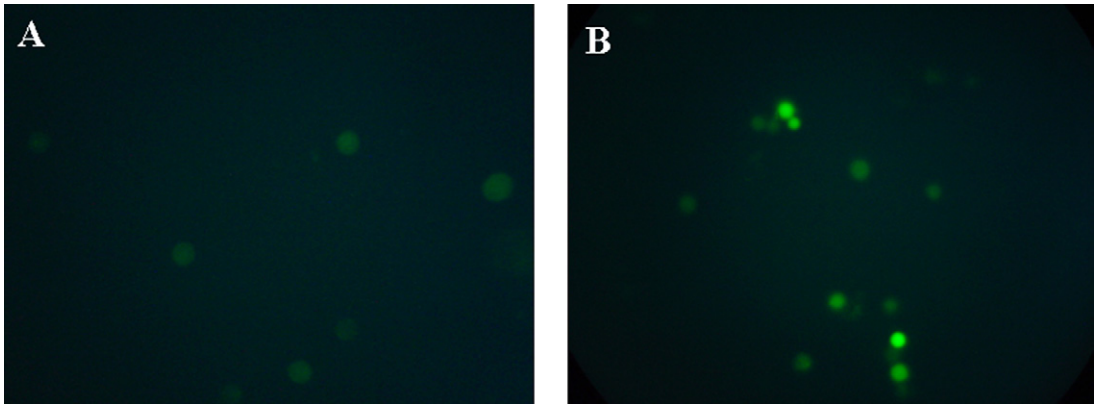

Fig. 3. Changes (expressed as differences in fluorescence intensity) of the cytoplasmic calcium ion concentration in N2a cells treated with pesticide free tomato samples (A) and tomato samples containing 14 different pesticides (B).

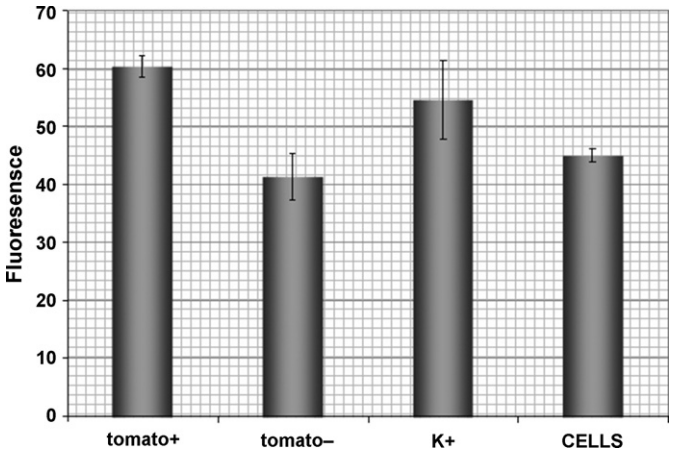

Fig. 4. N2a cells were treated with pesticide-free tomato samples (tomato −), tomato samples containing 14 different pesticides (tomato +) or K^+ solution (70 mM) (K^+). Cells treated with buffer were used as control (CELLS). Calcium mobilization was determined using a fluorometric imaging plate reader. Shown are average values ± standard deviations of triplicate determinations of endpoint measurements. These graphs are representative of four similar experiments.

sor gave satisfactory results due to the high reproducibility and the differentiation between control and positive samples.

Vero cells are widely used in studies of in vitro cytotoxicity [31]. The results of the present study, as expected, indicate that Vero cells present higher specificity to the dithiocarbamate fungicide, propineb than to the organophosphate insecticide diazinon. These results provide an indication that the zinc organometallic fungicide propineb, reacts with the zinc receptors on the kidney cells [26]. Furthermore, N2a cells, as expected due to their membrane-bound AChE [32], demonstrate higher selectivity to the organophosphate diazinon in contrast to propineb (Fig. 1A). However, propineb resulted in a satisfactory response between the concentrations 173 nM and 3 nM (the latest being the lower detectable concentration). In previous studies using rat pheochromocytoma cells differentiated by NGF (PC12), propineb (0.001–100 nM) dose-dependently increased ACh release from PC12. At concentrations between 0.001 and 1 nM propineb-induced ACh release reached a maximal effect (~50%) while at higher concentrations (10–100 nM), propineb caused a progressive disappearance of the effect [21]. It seems that pesticide concentrations above a limit caused cellular toxicity or otherwise major, non-reproducible biochemical changes that could not fit in a linear response pattern. This effect was cell line- and pesticide-dependent, as observed by the results presented in Fig. 1, whereas cells responded to the applied pesticides in a linear pattern only within a certain range of concentrations.

The results of the present study showed a higher fluorescence intensity of the samples containing pesticides compared to pesticide-free samples. Calcium ions (Ca^{2+}) are universal secondary messengers that are key players in many cellular signal transduction pathways [17]. Cell damage induced by environmental insults or by overstimulation of physiological pathways results in pathological Ca^{2+} signals, which trigger necrotic or apoptotic cellular death [33]. Increased Ca^{2+} influx through voltage-operated calcium channels (VOCCs) is implicated in many forms of both apoptotic and necrotic cell death [34]. Wang and Xu [35] report a possible involvement of Ca^{2+} signalling in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells.

There are several areas of future work that would improve the utility of cell-based biosensors. Numerous applications would benefit from the development of parallel systems that allow for simultaneous measurements on multiple cell lines. The development of a system utilizing multiple cell types will offer improvements to both the breadth of sensitivity and the ability to discriminate or classify analytes.

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