Extracellular Bio-imaging of Acetylcholine-stimulated PC12 Cells Using a Calcium and Potassium Multi-ion Image Sensor

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In biochemistry, Ca^{2+} and K^{+} play essential roles to control signal transduction. Much interest has been focused on ion-imaging, which facilitates understanding of their ion flux dynamics. In this paper, we report a calcium and potassium multi-ion image sensor and its application to living cells (PC12). The multi-ion sensor had two selective plasticized poly(vinyl chloride) membranes containing ionophores. Each region on the sensor responded to only the corresponding ion. The multi-ion sensor has many advantages including not only label-free and real-time measurement but also simultaneous detection of Ca^{2+} and K^{+} . Cultured PC12 cells treated with nerve growth factor were prepared, and a practical observation for the cells was conducted with the sensor. After the PC12 cells were stimulated by acetylcholine, only the extracellular Ca^{2+} concentration increased while there was no increase in the extracellular K^{+} concentration. Through the practical observation, we demonstrated that the sensor was helpful for analyzing the cell events with changing Ca^{2+} and/or K^{+} concentration.

Keywords Bio-imaging, ion image sensor, multi-ion image sensor, polyvinyl chloride membrane, PC12 cell

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Introduction

In biochemistry and the life sciences, a deeper understanding of molecular behavior and signal transduction can lead to the development of new medicines and elucidation of the causes of various diseases. It is important to have information about biomolecules and ions, since their concentrations are closely related to biochemical reactions. However, it is impossible to see the changes in their concentrations directly. Therefore, much interest has been focused on the methods that facilitate understanding of cellular physiology, that is to say bio-imaging. Fluorescent imaging is one of the most commonly used bio-imaging techniques. With the development of fluorescent protein markers, detailed observation of events in living cells has been achieved by fluorescent imaging.¹

Meanwhile, there is growing demand for bio-imaging without the use of labeling reagents. The advantages of such imaging methods are that the measurement sample is kept free of impurities and it can be returned back to the living body after the observation. In recent years, a variety of chemical sensors featuring label-free biological detection have been studied intensively.² We also have been developing one of these label-free sensors, which is referred to as an ion image sensor.³

For neurophysiological measurements, chemical sensors that

particularly detect hydrogen ion concentration have been actively researched. It is well known that a semiconductor pH sensor based on ion sensitive field effect transistor (ISFET), which is quite durable compared with a conventional glass electrode, was introduced by Bergveld in 1970.⁴ A standard complementary metal-oxide semiconductor (CMOS) process was incorporated with the manufacture of ISFET in order to improve signal-to-noise ratio (SNR) and compensate for temperature.^{5,6} A highly sensitive ion sensor whose accumulated sensitivity is 450 mV/pH using a charge coupled device (CCD) process has been reported.⁷ Two-dimensional (2-D) pH imaging was achieved by combining the above CMOS image sensor with the CCD process.⁸ Such 2-D imaging is useful for analyzing information about locally changing ion concentration. With the advancement of integrated circuit technology, spatial resolutions of these pH image array sensors have been steadily improving: a 32×32 pixels array sensor was developed in 2007,⁹ a 128×128 pixels sensor in 2013,¹⁰ and a 1024×1024 pixels sensor in 2016.¹¹ A CCD-type pH image sensor coated with a plasticized poly(vinyl chloride) (PVC) membrane containing an ionophore can be used as an ion image sensor. In biological reactions, both hydrogen ion and various cations play major roles. We have developed ion image sensors that can detect these important ions in living organisms, such as potassium ion (K^{+}), sodium ion (Na^{+}), and calcium ion (Ca^{2+}).¹²⁻¹⁴ As an example of the practical measurement using a K^{+} image sensor, glutamate-induced changes in K^{+} concentration were monitored in hippocampal slices.¹⁵ Recently, a Na^{+} - K^{+} multi-ion image

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sensor capable of measuring two kinds of ions simultaneously was developed.¹⁶ In addition, we have also reported neurotransmitter image sensors based on the same CCD-type pH image sensor technique, such as histamine,¹⁷ acetylcholine (ACh),¹⁸ and adenosine triphosphate (ATP)¹⁹ image sensors.

In this paper, the extracellular concentrations of both Ca^{2+} and K^{+} on PC12 cells were monitored in order to demonstrate the practical usefulness of a Ca^{2+} - K^{+} multi-ion image sensor. We already reported the fabrication of the Ca^{2+} - K^{+} multi-ion image sensor in 2016.²⁰ In this previous work, we confirmed that the sensor had fast real-time response and high selectivity for target ions through the observation of stimulated HeLa cells, which led to the leakage of K^{+} from intracellular fluid. In order to prove that the multi-ion sensor had wide application potential for both Ca^{2+} and K^{+} , a cell event in which Ca^{2+} was strongly related to the dynamics was monitored in contrast to the previous observation that was mainly associated with the dynamics of K^{+} .

As a target for bio-imaging, we selected rat pheochromocytoma (PC12) cells, which have been widely used in neurobiology and neurochemistry since they were established in 1976.²¹ PC12 cells treated with nerve growth factor (NGF) differentiate into sympathetic neuron-like cells, and they are extensively known as a model system for neurosecretion.²² Neuronal nicotinic ACh receptors (nAChRs) respond to ACh, and work as ligand-gated ion channels in PC12 cells. In addition, there are other ion channels called voltage-operated Ca^{2+} channels (VOCCs) that are opened by depolarization following the activation of nAChRs.²³

For multi-ion imaging of PC12 cells, we statistically analyzed the potential responses of all sensing pixels by making use of the characteristics of the sensor array. Such statistical analysis is quite useful, especially when the size of an observation object is quite small such as living cells. When PC12 cells exposed to NGF for six days were stimulated by an ACh solution, the changes in ion concentrations accompanying the stimulation were successfully detected by the multi-ion sensor. The observation showed that the ACh-induced increase was an event specific to Ca^{2+} , in other words, there was no significant increase in the level of K^{+} . By monitoring two kinds of ions simultaneously, we could obtain clear evidence that plasma membranes of PC12 cells did not collapse throughout the measurement. The Ca^{2+} - K^{+} multi-ion image sensor is expected to be useful for simultaneously analyzing extracellular dynamics of Ca^{2+} and K^{+} on living cells.

Experimental

Reagents

1-[2-Amino-5-(2,7-difluoro-6-acetoxymethoxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoxymethyl) estersodium (Fluo-4 AM), tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (Na-TFPB) and 2-nitrophenyl octyl ether (NPOE) were purchased from Dojindo Lab., Inc. (Kumamoto, Japan). Cyclohexanone (CHN), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DME/F-12), 10,19-bis[(octadecylcarbamoyl)methoxyacetyl]-1,4,7,13,16-penta-oxa-10,19-diazacycloheicosane (K23E1), 1,3,5,7,9,11,13,15-octa(propyl methacryl)pentacyclo[9.5.1.13.9.15.15.17.13] octasiloxane (POSS), poly(vinyl chloride)/low molecular weight (PVC), tris(hydroxymethyl) aminomethane (Tris), valinomycin and acetylcholine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Potassium tetrakis(4-chlorophenyl)borate (K-TCPB) was purchased from Alfa Aesar (Ward Hill, MA), and 2.5S

Table 1 Composition of calcium ion selective membrane

Material	Mass/mg
PVC	30.0
POSS	10.0
NPOE	55.0
K23E1	3.6
Na-TFPB	1.4

Table 2 Composition of potassium ion selective membrane

Material	Mass/mg
PVC	30.0
POSS	30.0
DOS	36.0
Valinomycin	2.7
K-TCPB	1.3

Nerve Growth Factor (NGF) was purchased from BD Biosciences (San Jose, CA, USA). Bis(2-ethylhexyl) sebacate (DOS), stabilizer free tetrahydrofuran (THF) and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pure water used in all experiments was prepared by a Milli-Q system.

Preparation of Ca^{2+} - K^{+} multi-ion image sensor

The Ca^{2+} - K^{+} multi-ion image sensor was prepared from a CCD-type 128×128 pixels array pH image sensor.¹⁰ The surface was Si_3N_4 , and it required pretreatment in order to enhance adhesion between the surface and a plasticized PVC membrane. The sensor was soaked in pure water for an hour, subsequently soaked in 0.1 mol/L (M) of NaOH solution for half an hour, then rinsed with pure water, and dried. Both Ca^{2+} selective membrane and K^{+} selective membrane were prepared by dissolving the base material, nanofiller, plasticizer, ionophore and anion exclusion agent in a mixture solvent, 5 mL of THF and 5 mL of CHN. The compositions of ion selective membranes are listed in Tables 1 and 2. These ion selective membranes were used as printing solutions for inkjet printing. Inkjet printing is suitable for preparing a multi-ion image sensor since this method is capable of precisely controlling the size of droplets and the printing area.¹⁶ The upper half of the sensing area was coated with the Ca^{2+} selective membrane, and the other half was coated with the K^{+} selective membrane using a piezoelectric dot inkjet printer, IJK-200T (Microjet, Shiojiri, Japan) with a nozzle diameter of 300 μm (IJHB-300). Voltage parameters were adjusted as follows: pulse voltage 70 – 80 V, 1st pulse width 6.0 – 10.0 μs , interval pulse width 4.0 – 8.0 μs , 2nd pulse width 6.0 – 10.0 μs , and frequency 500 Hz. Under such conditions, the printing solutions were discharged and repeated printing was 20 times. The film thickness value was approximately 2 μm .¹⁶ After printing, the sensor was dried for about 24 h at 60°C to completely remove the solvent.

Preparation of PC12 cells

PC12 cells were grown on a poly-L-lysine-coated (PLL) 60 mm dish (Asahi Glass Co., Ltd, Tokyo, Japan), and incubated at 37°C with 5% CO_2 in an incubator. Growth medium consisted of DME/F-12 with 10% FBS, 100 units/ml of penicillin G and 100 $\mu\text{g}/\text{mL}$ of streptomycin. When the cells reached approximately 80% confluence, they were split into a collagen

vitrigel membrane having a thickness of 15–20 μm (CVM) (AGC Techno Glass Co., Ltd., Shizuoka, Japan), and 50 ng/mL of 2.5S NGF was added to the cells. After the addition of NGF, the cells gradually grew into sympathetic neuron-like cells. PC12 cells exposed to NGF completely differentiate, and extend neurites in six days.²⁴ Fluo-4 AM²⁵ was used for Ca^{2+} imaging in order to ensure that the PC12 cells incubated with NGF grew enough to respond to an ACh stimulus. When an ACh solution (final conc. 100 μM) was injected into the cells on the CVM with a micropipette, the level of intercellular Ca^{2+} in the cells increased steadily. It indicated that the cultured PC12 cells were affected by NGF properly, and they were activated by the ACh stimulation. Fluorescence images of intercellular Ca^{2+} were obtained by a digital microscope camera, DP80 (Olympus, Tokyo, Japan).

Multi-ion imaging of ACh-stimulated PC12 cells

Six days after the splitting into a CVM, Ca^{2+} - K^{+} multi-ion imaging of the cultured PC12 cells was carried out. The CVM with the cells was moved to the sensor surface with tweezers. The sensor was filled with 180 μL of the growth medium including 0.946 mM of Ca^{2+} and 3.76 mM of K^{+} . Following the move of the CVM including PC12 cells, 20 μL of an ACh solution (final conc. 100 μM) was slowly added into the cells on the sensor with a micropipette in order to prevent the CVM from moving on the sensor. Finally, the potential response was monitored by the sensor for 300 s. For the measurement, a leak-less Ag/AgCl electrode of LF-1 (BioResearch Center, Nagoya, Japan) was used as a reference electrode.

Results and Discussion

Evaluation of Ca^{2+} - K^{+} multi-ion image sensor

In our previous examination of the Ca^{2+} - K^{+} multi-ion image sensor,²⁰ both ion selective membranes responded to only the primary ion, and we demonstrated a successful simultaneous imaging of both ions. When the high concentration of Ca^{2+} or K^{+} solution was instantly injected into the sensor, each concentration profile changed completely within 1 s. Here, we statistically analyzed the potential responses of all sensing pixels. Figure 1a shows the histogram of potential difference from the baseline potential with the Ca^{2+} concentration varying from 0.1 to 10 mM in tenfold steps. After the sensor was rinsed with the same sample solution several times, the output signals were obtained at 90 s. A blank solution containing 1.0 mM of K^{+} without Ca^{2+} was measured to determine the baseline potential. This was followed by measuring the sample solutions with known Ca^{2+} concentrations, namely, 0.1, 1.0, and 10 mM. The values of potential were quite stable during the measurement to 90 s. Throughout these sensing experiments, the K^{+} concentration was fixed at 1.0 mM in order to precisely compare each potential response with the baseline potential. The average and the standard deviations are plotted in Fig. 1c in blue and as error bars, respectively. The potential slope for the average values of Ca^{2+} was 22.3 mV/decade. The readout potential of each sensing pixel depended upon the sensing properties of pixels, including a source follower gain, a transfer gain, and membrane potential.¹² Therefore the potential slope was an apparent value, and did not match with the membrane potential. As an evaluation from the response to pH, 26.4 mV/decade was obtained as the membrane potential. The value did not reach 29.6 mV/decade of the Nernstian response, although it resulted from the optimal membrane contents listed in Table 1. The nanofiller of POSS was useful to keep the membrane from

Fig. 1 (a) Histogram of potential difference against calcium ion concentration. (b) Histogram of potential difference against potassium ion concentration. (c) Average sensitivity to calcium ion and potassium ion. Error bars represent standard deviations of 6400 pixels.

peeling off, however, it reduced the apparent potential slope.

Similarly, Fig. 1b shows the histogram of potential difference against the K^{+} concentration. The baseline potential was measured from a blank solution containing 1.0 mM of Ca^{2+} without K^{+} . Subsequently, the sample solutions with known K^{+} concentrations, 0.1, 1.0, and 10 mM, were introduced to the sensor in turn. The potential slope for the average values of K^{+} was 51.0 mV/decade, and the average is plotted in Fig. 1c in red. The membrane potential of the K^{+} membrane was calculated at 60.2 mV/decade from the evaluation of pH response. The value almost corresponded with the Nernstian response.

As shown in Fig. 1c, the working curves revealed that 1 mM of each coexistence ion did not affect the sensor response to

Fig. 2 Calcium-potassium multi-ion imaging pictures of stimulated PC12 cells. Voltage scale: -10 mV (red), 0 mV (green), 10 mV (blue), and dead pixels (black). Stars represent the position of pixels where real-time responses are shown in Fig. 3.

measure Ca^{2+} and K^{+} in their concentrations of $0.1 - 10$ mM. In addition, the working curve indicated a limit of measurement, which was calculated from extrapolating the linear relations in 1 mM of a coexistent ion solution. From the intersections with zero potential difference, the limit values for K^{+} and Ca^{2+} were 0.02 and 0.007 mM, respectively. In the present examination of PC12 cells, the concentration of Ca^{2+} was *ca.* 1 mM, and the concentration of K^{+} was *ca.* 4 mM. Thereby the interfering effect of Ca^{2+} on the K^{+} membrane can be neglected clearly. On the other hand, the interfering effect of K^{+} on the Ca^{2+} membrane is estimated by the limit value from the Ca^{2+} working curve. Based on general considerations of selectivity coefficient for an ion sensor by the mixed solution method,²⁶ the limit value for Ca^{2+} was calculated at 0.1 mM in 4 mM of K^{+} solution. Consequently, the multi-ion sensor covers a wide range of their concentrations from 0.1 to 10 mM, which is relevant to the present PC12 cell events. In addition, each region on the sensor responded to only the corresponding ion.²⁰ Such a wide detectable concentration range and selectivity are absolutely necessary for the simultaneous detection of multiple ions.

Multi-ion imaging results of ACh-stimulated PC12 cells

Extracellular Ca^{2+} - K^{+} multi-ion imaging pictures are shown in Fig. 2. Each picture was processed by subtracting the reference value before the stimulation from the measured value. The upper half region was coated with the Ca^{2+} selective membrane, and the lower half area was coated with the K^{+} selective membrane. The ACh solution was slowly added at around 25 s, and the monitoring of surface potential continued to 300 s. Before starting the stimulation, the sensor potential was constant to 25 s. The reference potential is represented in green, while an increase and a decrease in potential correspond to blue and red, respectively, in Fig. 2. The cells were located between the ion sensitive membranes and the CVM. The stimulus, ACh, was carried by convection along the surface of the CVM rather than passing through the CVM directly. It is difficult to predict from which the solution begins to spread in advance due to an influence of convection by the injection of the ACh solution. In this experiment, the stimuli solution apparently started to spread

Fig. 3 Real-time responses of potential difference after stimulation to PC12 cells. (a) Calcium ion region, (b) potassium ion region.

from the lower right part of the imaging pictures in the K^{+} region, and subsequently spread to the upper area in the Ca^{2+} region. The extracellular bio-imaging pictures could offer information to intuitively understand the cell events happening. The apparent large change in the potential of the K^{+} region rather than the Ca^{2+} region reflects the potential slope based on the ionic valence.

Figure 3 shows the real-time responses at pixels of the Ca^{2+} region and the K^{+} region. The increase in potential was observed at a pixel in the Ca^{2+} region, and the potential decrease was observed at a pixel in the K^{+} region. Statistical analysis complemented the bio-imaging results, and facilitated further understanding of the phenomenon observed. Figures 4a and 4b show the histograms of potential difference of all sensing pixels after the stimulation. In addition, the histogram in the Ca^{2+} region without the cells is shown in Fig. 5 as a negative control. As can be seen from Fig. 3, the potential difference curves to time have the deviation due to noise, however, the noise was not synchronizing at each pixel. Therefore, the histograms included their noise deviations. The potential change in the K^{+} region was approximately -3.7 mV on average at 300 s under the

Fig. 4 (a) Histogram of potential difference in calcium ion region after stimulation to PC12 cells. (b) Histogram of potential difference in potassium ion region after stimulation to PC12 cells.

presence of the cells as shown in Fig. 4b, while the decrease of -3.5 mV on average was observed in the case without the cells in the K^+ region (data not shown). There is no significant difference between these decreases. It was confirmed that the ACh stimulation to PC12 cells did not affect the K^+ concentration. The growth medium was slightly diluted by the addition of the ACh solution, so the concentration should come to 0.9 times. If the growth medium reached a homogeneous concentration of 0.9 times of the initial solution, the potential changes on average in the K^+ region would finally decrease to -2.3 mV. Although the observed potential changes slightly exceeded the calculated value, those decreases could be mainly attributed to dilution. Meanwhile, there was no significant increase of potential difference in the K^+ region. This provided the evidence that plasma membranes of PC12 cells did not break after the stimulation. If they had broken during the measurement, the level of K^+ should have increased according to the concentration gradient between extracellular fluid and intracellular fluid. The intracellular level of K^+ is roughly two orders of magnitude higher than its extracellular level.²⁷

Figure 4a shows the histogram of potential difference in the Ca^{2+} region after the stimulation. The decrease derived from dilution should be also found in the Ca^{2+} region similarly to the K^+ region. However, some part of pixels increased their responses, and the Ca^{2+} histogram began breaking into two peaks with the lapse of time. The origin of the movement toward a decrease was the dilution as stated above, while the extracellular increase in the level of Ca^{2+} which was relevant to

Fig. 5 Histogram of potential difference in calcium ion region after addition of ACh solution without PC12 cells.

another peak was clearly attributable to PC12 cells. In fact, the Ca^{2+} histogram had just one peak in the case of the absence of the cells as shown in Fig. 5. The increase of intracellular Ca^{2+} concentration was confirmed from the fluorescent imaging. The intracellular increase is associated with nAChRs. It is well known that activated $\alpha 7$ nAChRs leads to an increase in intracellular Ca^{2+} level by coupling to Ca^{2+} -induced Ca^{2+} release (CICR) from intracellular stores.²⁸ Therefore, it was assumed that the extracellular Ca^{2+} concentration increased due to the discharge. Since no increase in the level of K^+ was observed, it was suggested that the Ca^{2+} discharge was detected through Ca^{2+} channels, through which only Ca^{2+} was able to pass.

The final Ca^{2+} concentration at the surrounding such pixels was estimated to be approximately 1.23 mM from the initial concentration (0.946 mM) of Ca^{2+} since the peak position of the increase proved to be about 2.5 mV as shown in Fig. 4a. Consequently, the increase of 2.5 mV correspond to approximately 1.30 times the initial concentration. Nakanishi *et al.* demonstrated that the concentration of cytoplasmic Ca^{2+} in PC12 cells increased after ACh stimulation using fluorescence imaging.²⁹ In that study, the fluorescence intensity of the soma of the PC12 cell increased from about 60 to 80% under the following conditions: the concentration of ACh was 0.5 mM, and PC12 cells were cultured with NGF (50 ng/mL) for three days. In comparison with the previous result, it is reasonable to consider that the increase we observed in the Ca^{2+} region is due to ACh-stimulated PC12 cells.

It was confirmed that a combination of bio-imaging and statistical techniques was highly compatible with the analysis of small targets such as living cells. In addition, we repeated the same experiments from the preparation for the cells in order to verify the reproducibility of the cell events. As a result, the same tendency was observed in every experiment, namely, the decrease in the level of K^+ and the increase in the level of Ca^{2+} were detected. One of the advantages of multi-ion imaging is that each ion response can be easily compared with one another. It is able to provide clear evidence that a response to a stimulus is relevant to only a specific ion. In this observation, it revealed that Ca^{2+} moved through not broken plasma membranes but Ca^{2+} channels. Through multi-ion imaging experiments, we demonstrated the practical usefulness and biocompatibility of the Ca^{2+} - K^+ multi-ion image sensor.

Conclusions

We demonstrated the practical usefulness of a Ca^{2+} - K^{+} multi-ion image sensor featuring label-free and real-time measurements. Two different ion sensitive membranes on the multi-ion sensor were prepared using an inkjet printing method. The multi-ion sensor can detect Ca^{2+} and K^{+} simultaneously. As a result of the evaluation of the sensor, each plasticized PVC membrane containing a proper ionophore had sensitivities, 22.3 mV/decade to Ca^{2+} and 51.0 mV/decade to K^{+} within 0.1 – 10 mM. PC12 cells treated with NGF were used as the target for multi-ion imaging. The extracellular bio-imaging pictures and their real-time responses revealed that the Ca^{2+} concentration increased after the stimulation of ACh without the increase in the K^{+} region. These imaging results were helpful for intuitively understanding the cell events. Furthermore, we analyzed the sensing results statistically, and proved that the Ca^{2+} histogram began breaking into two peaks as time passed. In addition to intuitive understanding of the events, detailed information about them was obtained by combining bio-imaging with statistical analysis. We confirmed the practical usefulness of the sensor such as wide detectable concentration range, label-free measurement, statistical analysis, and biocompatibility through the bio-imaging of ACh-stimulated PC12 cells. We concluded that the multi-ion sensor was helpful for analyzing the cell events by changing Ca^{2+} and/or K^{+} . The Ca^{2+} - K^{+} multi-ion image sensor would be expected to help elucidate the mechanism of signal transduction in living cells.

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