Development of a Membrane Potential Assay to detect Sodium Channel Toxins

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Abstract:

The FLIPR membrane potential (FMP) dye (Molecular Devices) is a high throughput optical screening tool for cell based assay. A membrane potential assay was designed to detect sodium channel toxins. This study aimed to develop a membrane potential assay to detect Na+ channel toxins, by using FLIPR membrane potential dye to monitor changes in Na+ flux. Neuroblastoma SH-SY-5Y cell line and fluorescence plate reader (Tecan) was involved in this study. the assay was set, by finding the best FMP dye form (red or blue), the best 96 wells microplate in terms of its color (clear or black microplate) and the optimum cell density that give an accurate fluorescence signal. Testing validation of designed assay by sodium channel activators; KCL and veratridine, in addition to sodium channel blockers; tetradotoxin and procainamide hydrochloride, investigating effect of Indian red scorpion venom on sodium channel, to find out whether it is activation or inhibition. The assay detected procainamide hydrochloride effects on membrane potential. However, it appeared that the assay was less sensitive to tetradotoxin. That could be due to concentrations of provided TTX stock solution. Effects of Indian red scorpion venom (Mesobuthus tamulus) were successfully investigated by the assay. The venom has activator effects, as it showed an increase in fluorescence that was related to persistent activation of sodium channel and delay of its inactivation. Treatment of SH-SY-5Y neuroblastoma cell line with Indian red scorpion venom followed by veratridine, caused much more fluorescence increase than treatment of the cells with the venom only .The assay successfully detected sodium channels activator toxins such as veratridine and sodium channels blockers toxins such as procainamide hydrochloride. Activity of Indian red scorpion venom (Mesobuthus tamulus) investigated showed that the venom contains sodium channel activators.

Key: FLIPR membrane potential, tetradotoxine, veratridine, Sodium channels, Venom

Introduction

Voltage gated sodium channel functions through three functional stages; open state, resting state and inactivated state. When the membrane is not depolarized, sodium channels take the resting (closed) stage. Whereas, depolarization of the membrane activates these channels and shift them to the open stage, in which sodium ions rapidly enter the cell. This stage is followed by inactivation stage in which sodium influx decreases. After repolarization of the membrane sodium channels return to the resting (closed) stage (Clare *et al.*2000).

However, voltage gated sodium channels are identified as electrical signals regulating many biological and cellular events. For example, muscle contraction, hormones secretion, signals between the brain and other tissues, and the information translation process occurring in the brain. Moreover, in cellular level ion channels have effects on cellular events such as metabolism, gene expression and protein degradation and synthesis (Yu and Catterall, 2004). As a consequence of their important role in cellular control and signal transmission, ion channels are targeted by substantial numbers of biological toxins (Catterall *et al.*2007).

Voltage gated sodium channels are therapeutic targets. Studies of neurological disorders such as arrhythmia, pain, epilepsy and diseases resulting from ion channel genes mutations known as channelopathieshese diseases, allowed an opportunity to provide a reasonable understanding of the essential role of ion channels. In addition these studies contributed to improve drugs to be more specific and selective. There are many neurotoxins which target sodium channels and bind to their receptor sites on these channels (Cestele and Catterall, 2000). However, specificity and high affinity of neurotoxins leaded to take advantages of these properties, and suggested that neurotoxins are idealistic method to investigate the function and structure of sodium channels (Catterall *et al.* 2007).

Venom of the Indian red scorpion consists of: serotonin, hyaluronidase, mucous polysaccharides, enzyme inhibitors, and neurotoxic peptides. The neurotoxin peptides are the primary cause

of symptoms after scorpion sting. This is due to their interaction with ion channel, in addition to their destructive action on the nervous system (Petricevich, 2010). However, Bawaskar (1996) concluded that venom of the Indian red scorpion causes activation of sodium channels, resulting in over activity of neuron. Generally, each toxin from scorpion venom binds and acts on a particular receptor site (Horn and Rott, 1999). Scorpion toxins are classified into; Sodium channels toxins, Potassium channels toxins, Calcium channel toxins and chloride channel toxins. In addition, they are sub-categorized as scorpion α – toxins, scorpion β – toxins. Nonetheless, as the peptides of scorpion venom alter and modulate function of ion channels, these peptides have been used in cancer therapy, vaccines industry, and protein engineering (Petricevich, 2010).

Novel fluorescent dyes which show a high sensitivity to changes in membrane potential are used to monitor changes in membrane potential (Whiteaker *et al.* 2001; Gonzalez *et al*. 1999). Dyes which measure membrane potential alterations are divided into 2 groups: cyanine and axonal dyes with a slow respond and reasonable sensitivity to membrane potential alterations. Styryl dyes with a rapid respond and weak sensitivity (Zhang *et al.*1998). DiBAC4(3) and FLIPR membrane potential dye (FMP) are examples of the fluorescent dyes. The fluorescence of these dyes is extremely low in the extracellular environment. However, as soon as the dye bind to the membrane lipid, its fluorescence increases. When the cellular membranes are depolarized, the dye moves into the cytosol causing an increase in its concentration in the cells, and binding ratio to the membranes (Apell and Bersch, 1987). DiBAC4(3) is bis-(1,3-dibutylbarbituric acid) trimethine oxonol. It shows low response, and easily affected by temperature and dilution. In addition, its preparation requires several steps. Structure of (FMP) dye is unpublished. However, its response time is faster than DiBAC4(3). Moreover, FMP dye dose not require washing step, since the fluorescence is measured from the bottom of the well upwards (Baxter *et al.* 2002).

Related previous studies :

Wolff *et al.* (2003) developed a comparison study between four membrane potential probes, in which DiBAC4 (3) oxonol dye, and FMP dye were involved. They used RBL-2H3 cells (rat basophilic leukemia cells) which were seeded at different densities ranging from 10,000 cells/well to 50,000 cells/well into 96 microplates. Fluorescence signals were measured prior and after depolarization of the cells by (50 m M) KCL. They observed that both DiBAC4 (3) and FMP dyes indicated a linear relationship between the basal fluorescence and cell density before depolarization with KCL. After depolarization the FMP dye gave a maximum fluorescence signal at a density of 25,000 cells/well, and the signals remained stable until the cell density of 50,000 cells/well. Therefore, it was concluded that cell density from 25,000 to 50,000 cells/well can be used with FMP dye to give sufficient fluorescence signal. Louzao *et al.* (2000) used a fluorimetric assay depended on alteration in membrane potential to screen Paralytic Shellfish toxins. Paralytic Shellfish Poisoning (PSP) have leaded to deaths around the world. The representative of PSP toxins is saxitoxin (Shimizu, 1988). Saitoxin blocks neuronal transmission as it bind to the voltage gated sodium channel, and inhibit the generation of action potential (Narahashi, 1988). Nonetheless, PSP toxins were examined in terms of their ability to inhibit depolarization generated by veratridine. Different concentrations of saxitoxin were added after depolarization of the neuroblastoma cells by veratridine, and a decrease of induced depolarization was observed as a linear relationship between saxitoxin concentration and depolarization level (Louzao *et al.* 2000). According to the study of Catterall (1976), effect of scorpion toxins increased in the presence of veratridine giving rise to the sodium channel activation, and membrane action potential. The study used neuroblastoma cells to measure uptake of (22 Na+) under the influence of scorpion venom. Uptake of (22 Na+) increased slightly when the cells treated with scorpion venom alone. Whereas, addition of low concentration of veratridine generated higher increase in (22 Na+) uptake. Therefore, the study

suggested that scorpion venom poorly activates sodium channel, unless it followed by veratridine treatment. However, this activation inhibited by treatment of the cekks by (1 µM) tetrodotxin.

Study objectives :

Generally, this study aims to develop a membrane potential assay to detect Na+ channel toxins, by using FLIPR membrane potential dye to monitor changes in Na+ flux. Neuroblastoma SH-SY-5Y cell line and (Tecan) the fluorescence plate reader are involved in this study.

1-setting up the assay, by finding the best FMP dye form (red or blue), the best 96 wells microplate in terms of its color (clear or black microplate) and the optimum cell density that give an accurate fluorescence signal.

2-testing validation of designed assay by sodium channel activators; KCL and veratridine, in addition to sodium channel blockers; tetradotoxin and procainamide hydrochloride.

3- investigate effect of Indian red scorpion venom on sodium channel, to find out whether it is activation or inhibition.

Materials and methods :

Cell culture

SH-SY-5Y cells were grown in EMEM medium which is Eagle 'MEM (minimal Essential Media) supplemented with 10% Fetal Calf Serum, 15 penicillin/ streptomycin, 1% glutamine, and 1% non essential amino acids. Cell were maintained and grown into T-75's flasks with 20 ml of complete media into each flask, in a humidified incubator at 37 ^{O}C and 5% CO2 . neuroblastoma cells were sub cultured every 3-4 days.

Cell loading with membrane potential dye FMP

The FMP dye either blue or red was prepared as follow; 1 vial of the FMPdye was dissolved by (5 ml) of (1x Hanks' BSS buffer supplemented with 20 Mm Hepes, adjusted by HCL to 7.4), and then the vial was washed by another (5ml) of 1x Hanks 'BSS buffer. Therefore, (10 ml) of 1xHanks'BSS buffer was used to

dissolve the FMP dye. From the (10 ml)loading buffer, 20 x 500 µl aliquots were prepared and stored in (-20 ^{O}C).

In all the experiments cells were seeded overnight in 96- well microplates at volume of (100 µl). cells were loaded by the loading buffer directly after removing the microplates of the cells from the incubator. (100 µl) loading buffer was added to each well of the 96 well microplates, and incubated for 30 minutes at(37 ^{O}C) before each experiment. Fluorescence plate reader (Tecan) was used to measure fluorescence signal.

Chemicals and reagents

Scorpion venom stock solution (58mg/ml), KCL stock solution (1.5 M), veratridine stock solution (10 m M), tetradotoxin stock solution (3 m M), procainamide hydrochloride stock solution (500 m M).

Determination of the best 96 wells microplate, and the best FLIPR membrane potential dye

In order to compare clear and black microplates, blue and red (FMP) dyes in terms of giving the best fluorescence signals, neuroblastoma cells were seeded at a density of 50,000 cells/well in 96 clear and black microplates (100 µl) in each well. After incubation , cells in both microplates were loaded by red and blue dyes separately in each well. After cell incubation, fluorescence signals were recorded before and after addition of (100 m M) KCL, and (150 m M)KCL.

Preparing different cell densities

Cell densities of $1x10^5$, $2x10^5$, $3x10^5$, $4x10^5$, and $5x10^5$ were prepared , and cell counting was done by the hemocytometer. In a black 96 wells microplate, wells in the first column were left empty. While, wells in the second column filled by (100 µl) cell suspension that its cell density is $1x10^5$. similarly, wells in the third, fourth, fifth, and sixth column filled by (100 µl) cell suspensions that its cell densities/ml were $2x10^5$, $3x10^5$, $4x10^5$, and $5x10^5$ respectively. After incubation and cell loading with FMP red dye, the microplate was returned to incubator for (30 min) and

fluorescence signals were recorded each 10 seconds. KCL (150 m M) was injected to cell suspension in wells, in order to depolarize SH-SY-5Y cells. The fluorescence signals were recorded each 10 seconds for 50 seconds after injection of KCL.

Injection of KCL and Veratridine

Neuroblastoma cells were seeded at adensity of 50,000 cells/well in 96 black microplate. After incubation time cells were loaded by red dye (100 µl) for (30 min) at 37 ^{O}C in the incubator. Fluorescence signals were recorded before and after addition of KCL different final concentrations; (150 m M), (100 m M), (50 m M), and (30 m M). However, the previous steps were repeated , but cell suspensions were treated by veratridine instead of KCL. Veratridine final concentrations of (100 µM, 60 µM, 30 µM, 15 µM) were used to treat the cells. Fluorescence signals were recorded 20 seconds, and 10 seconds before injection of KCL and veratridine, and every 10 seconds for 50 seconds after injection of KCL and veratridine.

Injection of procainamide hydrochloride and tetradotoxin (TTX)

After cell seeding into a black 96 wells microplate, at a density of 50,000 cells/ well, and addition of loading buffer of red FMP dye and after incubation, Fluorescence signals were recorded. Veratridine final concentration of (100 µ M) was used to treat the cells, and change in fluorescence was recorded. Procainamide hydrochloride final concentrations of (30 µ M) and (60 µ M) were added to wells, and change in fluorescence was recorded. All previous steps were repeated , but Procainamide hydrochloride was replaced by tetradotoxin final concentrations; 300 n M, and 600 n M.

Injection of Indian red scorpion venom (Mesobuthus tamulus)

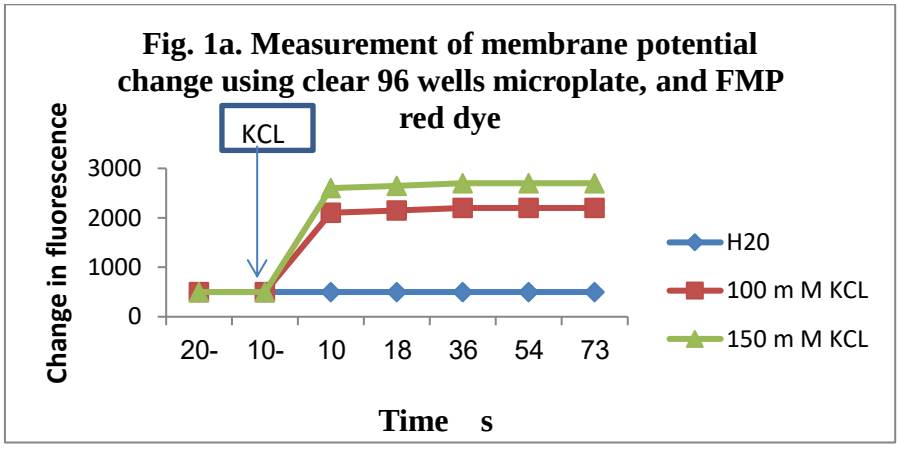
SH-SY-5Y cells were seeded into a black 96 wells microplate at a density of 50,000 cells/well, and addition of loading buffer of red FMP dye and after incubation, Fluorescence signals were recorded. Cells were treated by Indian red scorpion venom at final concentrations of (0.5 mg/ml), and (1mg/ ml) . change in fluorescence was recorded every 10 seconds five times. Veratridine

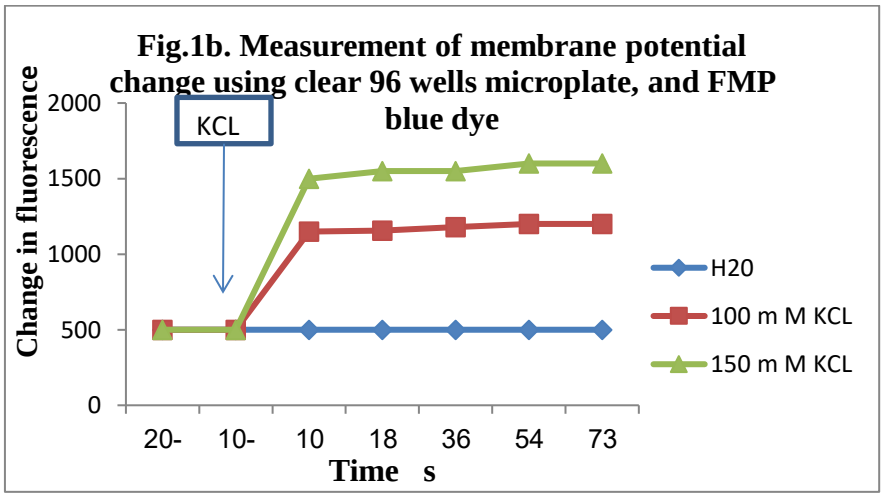
final concentration (100 µ M) was added to the wells injected by the venom, and change in fluorescence was recorded.

Results:

Measurement of change in membrane potential using clear 96 wells microplate and red, blue FLIPR membrane potential dye.

Cells loaded with red FMP dye showed a fluorescence signals that was approximately (2200, and 2700) after the injection of (100 m M, and 150 m M) KCL (fig. 1a). in contrast, cells loaded with blue FMP dye indicated lower fluorescence signals in comparison with red. As it indicated in (fig. 1b) cells after treatment with KCL showed fluorescence that was roughly (1600, 2200). Results were expressed as mean ±standard deviation (n =3).





Key: KCL (Potassium Chloride), s (second).

Measurement of change in membrane potential using black 96 wells microplates and red, blue FLIPR membrane potential dye.

Cells loaded with red FMP dye showed a fluorescence signal that was about (1000) prior depolarization, and approximately (4000, and 5000) after the injection of (100 m M, and 150 m M) KCL. On the other hand, cells loaded with blue FMP dye indicated lower fluorescence signals in comparison with the red. Before treatment of the cells with KCL, fluorescence was roughly (500). While after KCL treatment fluorescence was about (1800, 2100) as it shown in (fig. 2a and 2b). this confirms that red FMP dye is the best. However, fluorescence generated by FMP red dye using the black microplates was higher than that generated by the red dye using clear microplates (fig. 1a). results were expressed as mean ±standard deviation (n =3).

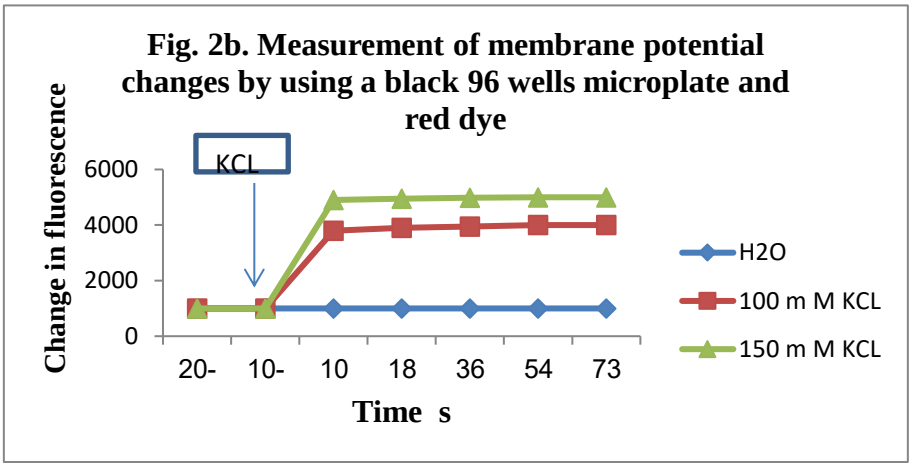


Fig. 2b. Measurement of membrane potential changes by using a black 96 wells microplate and red dye

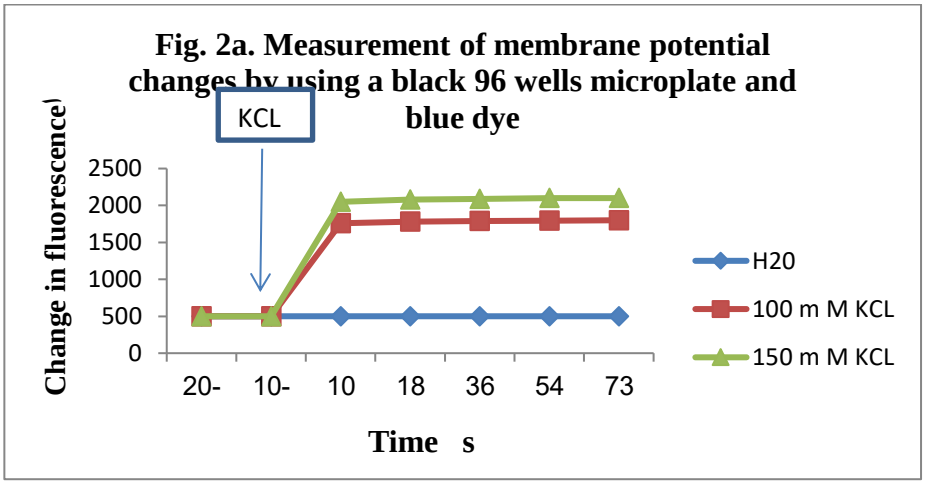
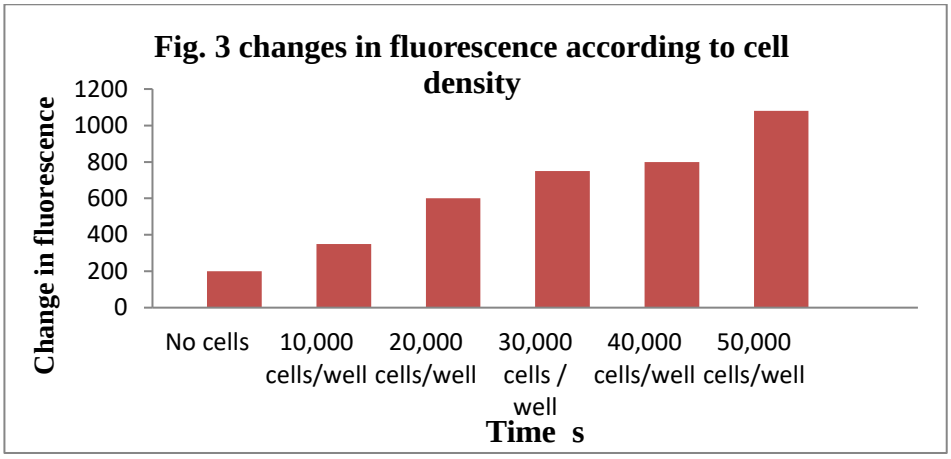


Fig. 2a. Measurement of membrane potential changes by using a black 96 wells microplate and blue dye

Key: KCL ((Potassium Chloride), s (second).

Determination of optimum cell density

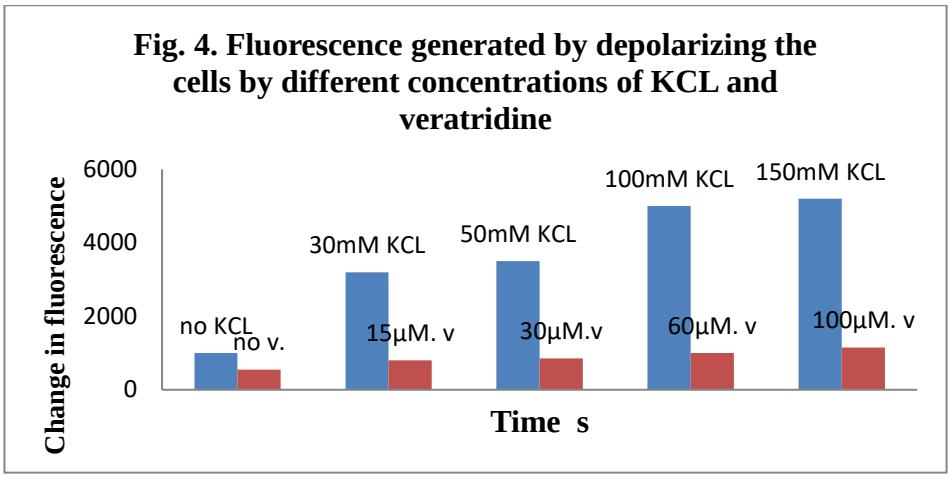
As it indicated in (fig. 3) cell density increase was accompanied with fluorescence signal rise. For example, cell density of 10,000 cells/ well indicated the lower fluorescence signal comparing with cell density of 50,000 cells/well, that indicated the highest fluorescence signal. Moreover, fluorescence signals increased rapidly maintaining difference in the signals between cell densities. Results were expressed as mean ±standard deviation (n =3).



Key: s (second).

Measurement of change in membrane potential after depolarization of the cells by KCL and veratridine.

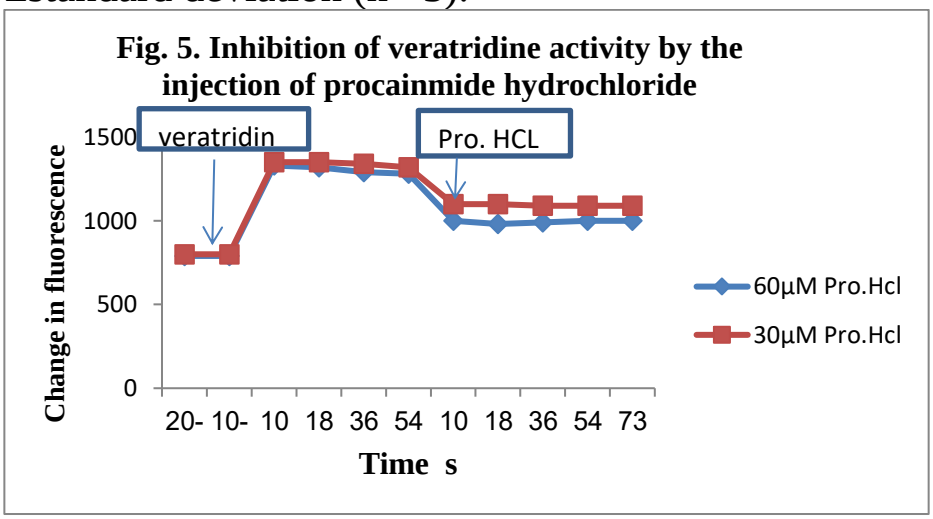
Fluorescence signals were measured before and after treatment of the cells by variable concentrations of potassium chloride KCL. Fluorescence signals increased dramatically after the injection of KCL (fig. 4). In addition, it can be seen that the increase in fluorescence signal was a concentration dependent. Change in membrane potential was measured prior and after depolarization of the cells by veratridine different concentrations . (fig. 4) demonstrates that veratridine generally caused a noticeable increase in the fluorescence signals. Similar to KCL, there was a linear relationship between fluorescence signals and veratridine concentrations. Results were expressed as mean ±standard deviation (n =3).



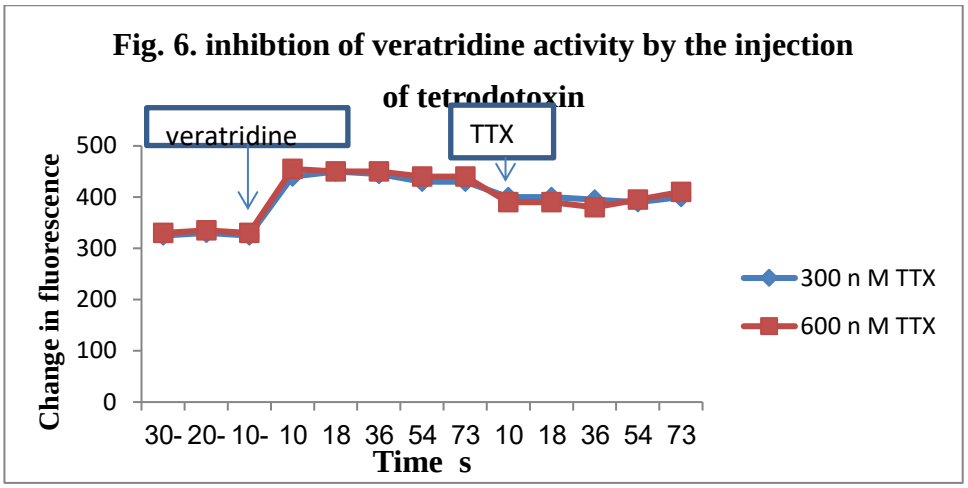
Key: KCL ((Potassium Chloride), v (Veratridine), s (second).

Measurement of change in membrane potential after hyperpolarization of the cells by procainamide hydrochloride (Pro.HCL) and tetrodotoxin (TTX).

Injection of (Pro.HCL) to the cells at final concentrations of (30 µM, and 60 µM), after treatment of the cells by (100 µM) veratridine inhibited fluorescence signals induced by veratridine leading to a decline in fluorescence as indicated in (fig. 5). Addition of (TTX) to cells depolarized by veratridine changed fluorescence signals. (fig. 6) demonstrates that addition of (100 µM) veratridine increased fluorescence signals. However, injection of (300 n M, 600 n M) decreased it. . Results were expressed as mean ±standard deviation (n =3).



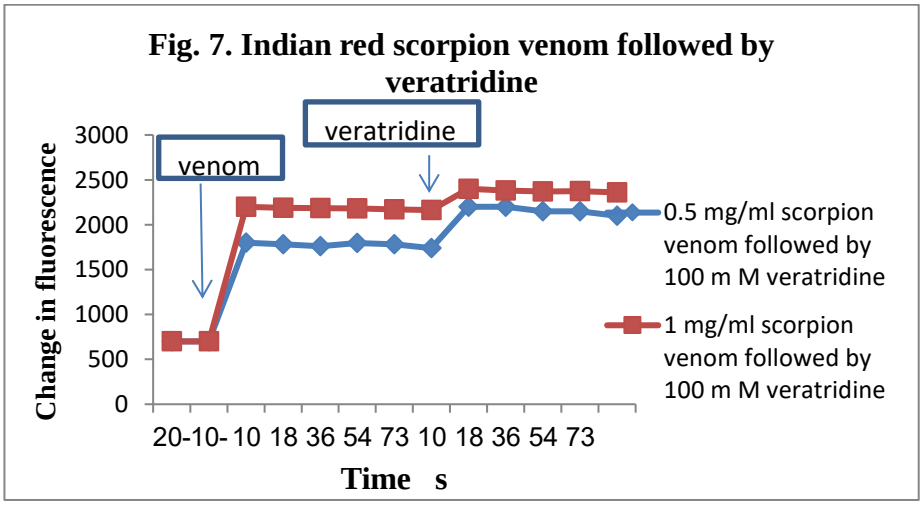
Key: s (second), Pro.HCL (Procaainmide Hydrochloride).



Key: TTX (Tetrodotoxine), s (seconds).

Investigation of Indian red scorpion venom activity

Injection of the Indian red scorpion venom depolarized the cells and leaded to increase fluorescence signals. Final concentrations of (0.5 mg/ml, and mg/ml) venom were sufficient to depolarize the cells. Since it caused a dramatic increase in the fluorescence. However, fluorescence was a concentration dependent. Treatment of the cells by (100 µM) veratridine after the addition of the venom gave rise to fluorescence and enhanced the activity of the Indian red scorpion venom as it illustrated in (fig,7). Results were expressed as mean ±standard deviation (n =3).



Key: s (seconds).

Discussion:

Setting up the assay

Cell density of 50,000 cells/well showed the highest fluorescence signals, which suggested this density to be used in the assay in all of the experiments. Injection of (150 m M) KCL generally increased the fluorescence maintaining differences between densities. These results agree with those of Wollf *et al.* (2003) as their work included determination of optimum cell density for different membrane potential dyes. In their work, they used rat basophilic leukemia cell line RBL-2H3, and seeded them in 96 well microplates. Basal fluorescence, and fluorescence after depolarization of the cells by (50 m M) KCL was reported. For FLIPR membrane potential dye. They found that 25,000 to 50,000 cells/well are suitable to work with this dye (Wolff *et al.* 2003).

FLIPR membrane potential dye (FMP), which is produced by Molecular Device Corporation, gives a rapid and reliable fluorescence based assay to detect alterations in voltage within the cell membrane. However, when the dye binds to the membrane lipid, its fluorescence increases. When the cellular membranes are depolarized, the dye moves into the cytosol causing an increase in its concentration in the cells, and binding ratio to the membrane (Apell and Bersch, 1987). Both dyes were compared in terms of giving best fluorescence signals, That was done by using both black and clear 96 wells microplates. Results confirmed that cells loaded with the red FLPIR membrane potential dye generated stronger fluorescence signals in comparison with those loaded with the blue dye. Nevertheless, structure of the FLIPR membrane potential dye, and the different between red and blue forms are unpublished.

Another comparative analysis was done between fluorescence given by cells seeded in black 96 wells microplates, and that given by cells seeded in clear 96 wells microplates using both red and blue (FMP) dyes. Results indicated that black microplates are better than clear microplates, as black microplates gave the best fluorescence signals. However, either blue or red (FMP) dye confirmed the same results. Consequently, it can be deduced that black frame decreases

signal crosstalk between wells. Study of (Klimovich *et al.* 2009) compared white, black, and clear microplates in terms of their properties including the color. It was found that the black color of the microplates decreases well to well crosstalk. Therefore, readings will be more accurate.

Testing validation of the assay with sodium channel activators

Potassium chloride KCL is known to depolarize cells and open sodium channels. Veratridine also causes similar effect by activating sodium channels. Thus, both reagent depolarize cells, and both were used to test the assay. Injection of increasing concentrations of KCL ranged from (30 m M) to (150 m M), produced increasing fluorescence signals, which were correlated with the changes of membrane potential as it shown in (fig. 4). These results agree study of (Baxter *et al.* 2002), which compared DiBAC4 (3) dye and FMP dyes in terms of their response to depolarization by different concentrations of KCL. it was concluded that FMP fluorescence related with changes of membrane potential increased according to KCL concentration increase indicating a strong linear relationship.

Moreover, veratridine concentrations from (15 µM – 100 µM) caused changes in FMP fluorescence correlated with membrane potential. These changes in fluorescence indicated a linear relationship between veratridine concentrations and fluorescence as it shown in(fig. 4). Ward (2002) investigated voltage gated sodium channel using molecular devices membrane potential assay kit, and several cell lines including SH-SY-5Y cell line. Veratridine concentrations (1 µM, 10 µM, 100 µM, 1000 µM) were used validate his assay. Ward observed that fluorescence signals increased as the concentration of veratridine increased, which was similar result to the current study.

Testing validation of the assay with sodium channel blockers

Tetradotoxin (TTX) and procainamide hydrochloride (Pro.HCL) are known as sodium channel blockers. Consequently, flu fluorescence should decline by the treatment of the cells by either tetradotoxin or procainamide hydrochloride. Injection of tetradotoxin at final concentrations of (300 n M, and 600 n M) to

the cells, caused a slight decrease in fluorescence, that was not significant. However, the reason could be due to the concentration of the provided tetradotoxin stock solution. Study of Ward (2002) assayed TTX in neuroblastoma cells lines including SH-SY-5Y cell line. His study examined effect of TTX on cells depolarized by

(100 µ M) veratridine, using the molecular device membrane potential assay kit. Results indicated that TTX caused a decrease in fluorescence that was a concentration dependent.

Injection of (Pro. HCL) at final concentrations of (30 µ M, and 60 µ M) to the cells leaded to noticeable decrease in fluorescence that was concentration dependent. For instance, (60 µ M) of Pro. HCL caused a dramatic decline in fluorescence in comparison with (30 µ M) of Pro. HCL.

Investigation of Indian red scorpion venom (Mesobuthus tamulus) effect on membrane potential.

As it indicated in result section, when the Indian red scorpion venom was added at final concentrations of (0.5 mg/ml, and 1 mg/ml) to the cells, fluorescence signals increased noticeably compared with the basal fluorescence line that was recorded before the addition of the venom. That was due to cell depolarization by the venom, causing activation of sodium channels. Therefore, the persistent activation leaded to influx of the red (FMP) dye causing a noticeable increase in fluorescence. Addition of (100 µ M) veratridine to the cells treated by the venom of Indian red scorpion, caused a further increase in fluorescence. This suggests that veratridine enhanced activation of sodium channels caused by the venom.

Results from study of Catteral (1976), which used venom of the North African scorpion Leiurus quiinquestriatus, support the previous observation. Uptake of 22 Na + by neuroblastoma cells was used to detect changes in membrane potential related to sodium channels. The study reported that treatment of the cells with scorpion venom only caused an increase in 22 Na + uptake rate. Whereas, the cells showed more 22 Na +uptake when they treated with veratridine after the treatment by scorpion venom.

Consequently, the study concluded that scorpion venom and neurotoxins such as veratridine work cooperatively to activate sodium channels.

Several studies examined effect of different scorpions venoms on action potential. Rowan et al. (1992) reported that Buthus tamulus (BT) venom at concentrations more than (20 mg/ml) leaded to depolarization. Moreover, study of Narahashi *et al.* (1972) concluded that (BT) venom caused prolongation of action potential by delaying inactivation of Na+ channels and blocking K+ channel. Bernard *et al.* (1977) tested effect of Androctonus australis venom on neuroblastoma cells. The study deduced that scorpion venom increased duration of the action potential of neuroblastoma cells.

Conclusion

A membrane potential assay designed to detect sodium channel toxins. Setting up the assay was by seeding SH-SY-5Y cells at a density of 50,000 cells/well in black 96 wells microplates, and loading the cells by red FLIPR membrane potential (FMP) dye. The assay successfully detected sodium channels activator toxins such as veratridine, and sodium channels blockers toxins such as procainamide hydrochloride. Activity of Indian red scorpion venom (Mesobuthus tamulus) was successfully investigated by the assay, and it was concluded that the venom contains sodium channel activators.

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