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Research Institute of Marine Science and Technology
Korea Maritime University

Mechanism of Na^+ transfer biosensor using a frog bladder cell membrane

J. S. Yoo,* B. S. Cheun[1] and Y. Seo[2]

Research Institute of Marine Science and Technology, Korea Maritime University, Busan 606-791, Korea
[1]Department of Molecular Biology College of Medicine Kyunghee University, Seoul 130-701, Korea
[2]Division of Ocean Science, Korea Maritime University, Busan 606-791, Korea

ABSTRACT

Gonyautoxin (GTX), saxitoxin (STX), and tetradotoxin (TTX), also known as paralytic shellfish poison (PSP) block Na^+ channels including those in the frog bladder membrane. The study on the direction of Na^+ transfer, in the absence of Na^+ channel blockers, revealed that active transport of Na^+ occurs across the frogs bladder membrane from the internal to the external surface or side. Transfer was shown to be STX-sensitive. The tissue sensor response to each of the different PSP was recorded and the results were compared with toxicities determined by the standard mouse bio-assay. In the present study, we investigated cell cycle arrest and increase of apoptosis to understand the antiproliferative effect of natural toxin of STX on various MTT assay cervical cancer cell lines.

Key words: Toxins, Na^+ channel transfer, cell membrane, cancer

1. Introduction

Among the Na^+ channel blocking toxins, paralytic shellfish poison (PSP) can be present in plankton and concentrated by filter feeding shellfish. PSP comprise gonyautoxin (GTX), saxitoxin (STX), and tetrodotoxin (TTX), and other related derivatives. They can block Na^+ channels in mammalian nerve cells, thus lead to paralysis upon consumption of contaminated animals. The biochemical characteristics, food safety, therapeutic implications of these toxins have been described (1). The observation that many Na^+ channels exist in the frog bladder membrane has allowed us to construct a tissue biosensor consisting of a Na^+ electrode covered with a frog bladder membrane for a simple and rapid electronic signal detection system for Na^+ channel blockers in cell membrane (2, 3). We established that, in the absence of Na^+ channel blockers, active transfer of Na^+ across the frog bladder membrane occurs from the internal to the external side of the membrane.

The presence of STX- sensitive epithelial Na^+ channels in the frog bladder membrane was demonstrated and confirmed by the lack of effect of inactivated toxin. Recently, this toxin was identified as a very potent antileukemic agent (4, 5).

It is well known that tumor development is not only from abnormal cell proliferation or inhibition of differentiation, but also from reduced cell death due to inhibition of apoptosis (2, 6, 7, 8).

* Corresponding author: Jong Su Yoo,
Tel: 051-410-4758, Fax: 051-404-3538,
E-mail: jsyoo@hhu.ac.kr

2. Materials and Methods

Preparation of frog bladder membrane The bull frogs, *Rana catesbeiana* were purchased at a local fish market. 10 mm×10 mm sections of frog bladder membrane were prepared and stored in 0.8% NaCl solution, containing 0.003% sodium azaid (NaN_3) to prevent microbial contamination.

Tissue biosensor system As shown in Fig. 1, the tip of the Na^+ electrode (a) was covered with the frog bladder membrane (c) sandwiched between two sheets of cellulose acetate membrane, (b) and (d). The cellulose acetate membrane protected the bladder membrane from the external solution. The internal surface of frog bladder membrane had to be opposite to the tip of the Na^+ electrode.

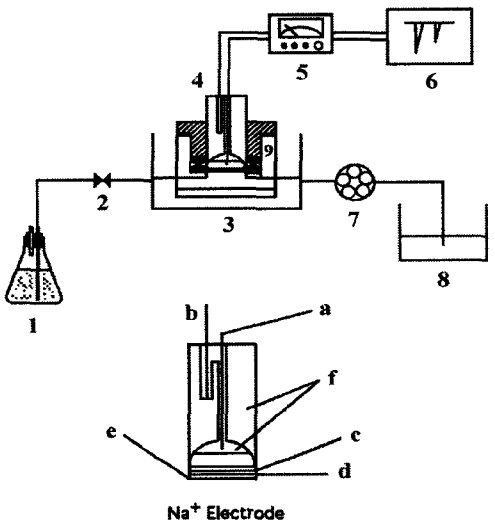


Fig. 1. Schematic diagram of the tissue biosensor flow system used for the determination of Na^+ channel blockers toxin. 1. 0.8% NaCl (pH 4.8) tank 2. injection part 3. thermostatically controlled bath 4. Na^+ electrode 5. electrometer 6. recorder 7. peristaltic pump 8. ion NaOH reservoir. (a) working electrode, (b) reference electrode, (c) cellulose acetate membrane, (d) frog bladder membrane, (e) cellulose acetate membrane, (f) 1M NH_4Cl.

This electrode was integrated within a flow cell connected to a peristaltic pump (6), injection port (2), and 0.8% NaCl tank (1). The flow cell was maintained at 30°C with a thermostatically controlled water bath (3). The output of the sensor was amplified with an electrometer (4) and then recorded (5). A 10 N NaOH reservoir was necessary for safe disposal of toxins after each injection at sample port (7) (8).

Column chromatography The GTX and STX extracts were separated into their respective components by column chromatography on activated charcoal and Bio-Rex70 (Biorad, California) (9). The STX extracts were initially separated into two fractions, neoSTX and these individual were obtained fractions by repeating the chromatography step.

Investigation of sodium ion transport across the frog bladder membrane The tissue sensor responses to increasing concentrations of Na^+ were measured in the absence of Na^+ channel blockers under three conditions: (a) with the internal surface of the frog bladder membrane next to the tip of Na^+ electrode; (b) with the external surface of the frog bladder membrane next to the tip of Na^+ electrode; (c) with Na^+ electrode covered with cellulose acetate membrane (no frog bladder membrane).

Effects of toxins (neoSTX) on growth inhibition of cervical cancer cell line We observed the dose-dependent effects of the toxins (neoSTX) on the proliferation of the cancer cell line using MTT assay for 5 days (8).

3. Results

Mechanism of Na^+ transfer across the frog bladder membrane

The fact that frog bladder membrane contains many Na^+ channels implies that the membrane cells can work as biological active substances. The rate of Na^+ transfer across the frog bladder membrane was investigated and the rate of the Na^+ transfer across the frog bladder membrane as Na^+ electrode sensor output was calculated from the graph of

Na^+ transfer against time. Facilitated diffusion of Na^+ with a maximum initial rate of 35 $\mu g\ cm^{-2}s^{-1}$, occurred when the external tip of the frog bladder membrane was direct contact with the surface of the Na^+ electrode. However, when the internal tip of the frog bladder membrane was indirect contact with the surface of the Na^+ electrode, the maximum initial rate of Na^+ transfer was only 8 $\mu gcm^{-2}s^{-1}$(Fig. 2).

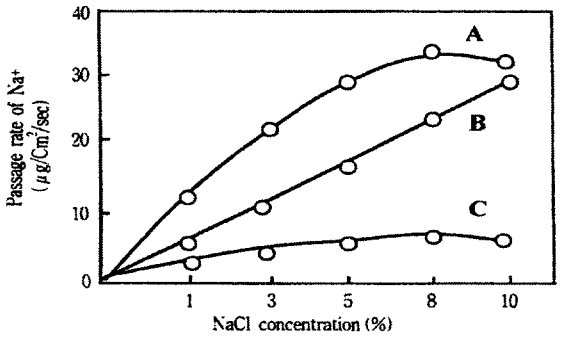


Fig. 2. Initial rate of Na^+ transfer across frog bladder membrane. Experimental condition, Temp 30℃, pH 4.8, flow rate 0.8 ml/min, sample volume 50 $\mu\ell$. (A) External surface of frog bladder membrane next to Na^+ electrode, (B) Internal surface of frog bladder membrane next to Na^+ electrode, (C) Na^+ electrode covered with cellulose ,acetate membrane (no frog bladder membrane).

In the absence of the frog bladder membrane, simple diffusion of Na^+ across the porous cellulose acetate membrane was seen using 0.3 to 1% NaCl solution with the external surface of the bladder membrane in contact with the Na^+ electrode the sensor output.

These results indicate that most STX-reactive sites are present on the internal surface of the membrane and facilitate Na^+ transfer, whereas only a few exist on the external surface, and that a route for Na^+ transfer not blocked by STX exists. These results are in agreement with those demonstrated that ADH- enhanced Na^+ transfer occurs only from the internal to the external surface of the toad bladder membrane (10).

neoSTX toxin f/mole/cell We observed the dose-dependent effects of the toxins (neoSTX,) on the proliferation of the cell line using MTT assay for 5 days neoSTX at exhibited 80% inhibition of growth in cell line (Fig. 3).

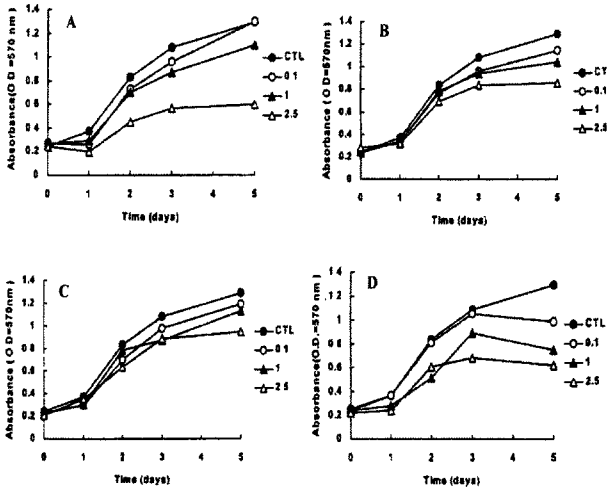


Fig. 3. Effect of toxin on growth inhibition of human cervical cancer HT3 cell line. 18 f mole/cell (4.97MU/106) (A), 43.9 f mole/cell (B), 70.95 f mole/cell (C), 80.96 f mole/cell (D).

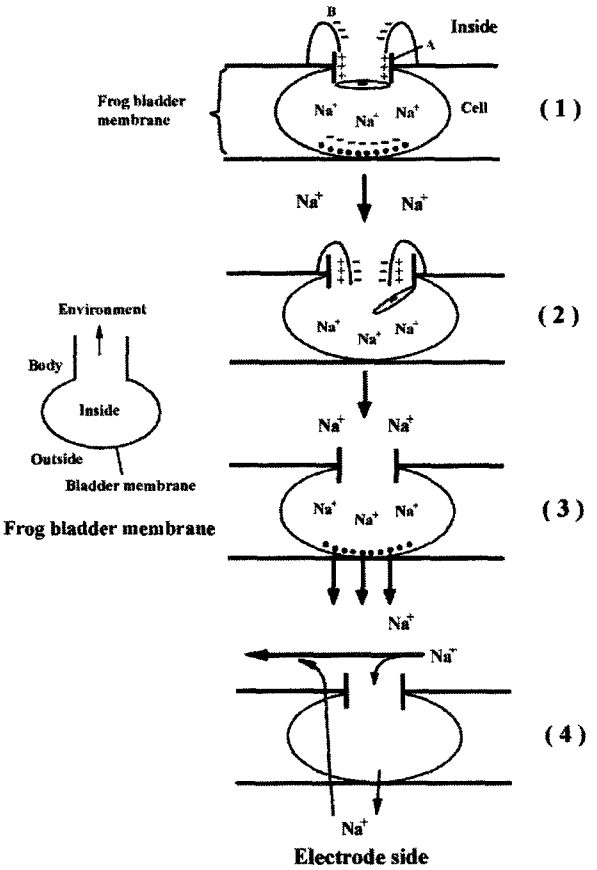
The mechanism of Na^+ transport across the frog bladder membrane in contact with the Na^+ electrode is explained in scheme 1 on the basis of the octagonal structure model of the Na^+ channel (11, 12, 13, 14).

The frog bladder membrane consists of about six layers of epithelial cells which readily adapts to the bladder filing and voiding. Scheme 1 is based on the assumption that the bladder membrane consists of a single layer of cells.

According to Sato *et al,* the Na^+ channel consists of a single protein chain of part A (called, S4) with + charge and part B (called, S5-6) with - charge c-terminal branch with - charge and N-terminal branch with + charge. Part A is inserted into the cell through the cell membrane and acts as arail for part B to slide into the cell. Part B generally works as the receptor of irritation (scheme (1)).

The exertion of force greater tham physiological threshed renders the bladder membrane in direct contact with the tip of the electrode and then 0.8 NaCl solution was transported. Namely, the potential difference between interior and exterior of cell membrane decreases and then part B slides in

to the cell along the rail 'part A' to push the lid 'C-terminal branches' aside. It seems that Na^+ flows in easily because the passage of Na^+ was facilitated by a little stretching (scheme 1 (2)).



Scheme 1. Mechanism of active Na^+ transfer across frog bladder ,membrane. 1. nonpolarised membrane 2. Application of gate-potential displaces electro protein and opens Na^+ channels, 3. Active transport of Na^+ ions at NaCl < 0.8% 4. Slow reverse transfer of Na^+ at NaCl > 0.8%.

The interior of the cell has a high concentration of Na^+ and the C-terminal branches no longer play the role of lid because the branches with the - charge are surrounded by Na^+ and are distant from N-terminal branches charged.

Most of the Na^+ pumps opposite the Na^+ channels in the interior of the cell are hardly working due to the of ATP. Therefore, Na^+ is transferred to the exterior of the cell by a simple diffusion(scheme 1(3)).

The Na^+ moved to the side of electrode has to transfer to the opposite side from the through the frog bladder membrane again.

It has been described that the one - way passage of Na^+ from the one side of the electrode to the opposite side seemed to exist on the frog bladder membrane. However as shown in scheme 1(4), a large flow of NaCl solution (0.8 ml/min) ran parallel to the membrane at the tip of the electrode.

It is assumed that Na^+ has been transferred by this flow. At this time when Na^+ channels have been blocked, the Na^+ concentration at the interior of the cell becomes momentarily. The diffusion rate of Na^+ from the interior to the exterior of the cell becomes the rate - determining step and the Na^+ concentration at the electrode side decreases. On the other hand, it is well known that Na^+ channel blockers combine with part B. It is described later that combined Na^+ channel blockers content agrees well with the passage content of Na^+.

However, the effect of Na^+ channel blockers on membrane Na^+ transport should be further examined in detail, by measuring the rate of Na^+ transfer in the presence of different concentrations of pure Na^+ channel blockers.

Na^+ channel blockers combined with part B up to a concentration of 0.3% NaCl and inhibited Na^+ transport, although at higher NaCl concentrations, the inhibitory effect decreased. These results indicate that Na^+ channel blockers dissociation is Na^+ concentration dependent. The presence of Na^+ channel blocker - sensitive Na^+ channels within the tissue biosensor was confirmed and the direction of Na^+ transfer was established. Also Na^+ channel blocker toxins (neoSTX) also inhibited the proliferation of cancer cell line in a dose- dependent manner.

4. Conclusions

The study on the direction of Na^+ transfer, in the absence of Na^+ channel blockers, revealed that active transport of Na^+ occurs across the frogs bladder membrane from the internal to the external surface or side.

In the present study, we investigated cell cycle arrest and reduction of apoptosis to understand the antiproliferative effect of natural toxin of STX on various MTT assay cervical cancer cell lines.

The presence of Na^+ channel blocker - sensitive Na^+ channels within the tissue biosensor was confirmed and the direction of Na^+ transfer was established. Na^+ channel blocker toxins (neoSTX) also inhibited the proliferation of cancer cell line in a dose-dependent manner.

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References

1. Bates, H.A. and H. Rapoport, 1975. A chemical assay for saxitoxin, the paralytic shellfish poison. J. Agric. Food Chem., **23**: 237-239.
2. Cheun, B., H. Endo, T. Hayashi, Y. Nagashima and E. Watanabe. 1996 Development of an ultra high sensitive tissue biosensor for determination of swellfish poisoning, tetrodotoxin. Biosens. Bioelectron, **11**: 1185-1191.
3. Cheun, B., H. Endo, T. Hayashi, K. Kim and E. Watanabe. 1997. Effect of storage conditions on the activity of the Na^+ channel existing on the frog bladder membrane. Nippon Suisan Gakkaishi, **63**: 616-620.
4. Chu, F.S. and T.S. Fan. 1985. Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. J. Assoc. Off. Anal. Chem., **68**: 13-16.
5. Davio, S.R. 1985. Neutralization of saxitoxin by anti-saxitoxin rabbit serum. Toxicon, **23**: 669-675.
6. Arakawa, O., T. Noguch, Y. Shida and Y. noue. 1994. Occurrence of carbamoyl-N-hydroxy derivatives of saxitoxin and neosaxitoxin in a xanthid crab *Zosimus aeneus*. Toxicon, ***32***: *175-183*.
7. Ferrari, F.K., T. Samulski, T. Shenk and R.J. Samulski. 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J. Virol., **70**: 3227-3234.
8. Ferrari, F.K., X. Xiao, D. Mc Carty and R.J. Samulski. 1997. New developments in the generation of Ad-free, high-titer rAAV gene therapy vectors. Nat. Med., **3**: 1295-1297.
9. Nagashima, Y., J. Maruyama, T. Noguchi and K. Hashimoto. 1987. Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high performance liquid chromatography. Bull. Japan. Soc. Sci. Fish., **53**: 819-823.
10. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson and H. Hamada, D. Pardoll and RC. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte- macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc. Natl. Acad. Sci. USA **90**: 3539-3543.
11. Hille, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. J. gen. physiol., **59**: 637-658.
12. Sato, C. and G. Matsumoto, 1992. Proposed terfiary structure of the sodium channel. Biochem. Biophys. Res. Commun., **186**: 1158-1167.
13. Sato, C. and G. Matsumoto. 1995. Sodium channel functioning based on an o-octagonal structure model. J .membr. Biol., **147**: 45-70
14. Tokuda, H. and T. Unemoto. 1985. The Na^+ -motive respiratory chain of marine bacteria. Microbiol. Sci., **2**: 69-71.