FOOD CHEMICAL CONTAMINANTS

Detection of Paralytic Shellfish Poison by Rapid Cell Bioassay: Antagonism of Voltage-Gated Sodium Channel Active Toxins in vitro

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Although cytotoxicity assays provide several advantages over mouse bioassays, sodium channel-blocking marine toxins, such as those associated with paralytic shellfish poison (PSP), require prolonged incubation periods of 24–48 h. This is in marked contrast to in vitro detection of sodium channel-enhancing marine toxins such as ciguatoxins or brevetoxins which can be accomplished in as few as 4–6 h. We developed a modified PSP cell bioassay that is as rapid as in vitro methods for sodium channel-enhancing toxins. The cell bioassay is based on a saxitoxin-dependent antagonism of the rapid in vitro effects of brevetoxin or ciguatoxin. Comparative analysis of naturally incurred PSP residues by both antagonism cell bioassay and the mouse bioassay demonstrated significant correlation. The simplicity, sensitivity, and enhanced kinetics of the new antagonism cell bioassay format provide the basis for development of a practical alternative to conventional mouse testing for PSP.

Monitoring of shellfish for marine toxins requires efficient test methods that can respond to toxins in proportion to total potency. The mouse bioassay has satisfied these requirements; however, ethical constraints, associated costs, and mandated restrictions have contributed toward the need for complementary and alternative bioassays. To address these concerns, a number of cell-based assays were developed for detection and analysis of marine toxins with activity at the level of the voltage-gated sodium channel. Cell bioassays for sodium channel-enhancing toxins, such as brevetoxins or ciguatoxins, are relatively rapid and can be accomplished in as few as 4–6 h (1–5). Interest in cell-based assays for marine toxins is evidenced by many requests for training at our facility (U.S. Food and Drug Administration, Bothell, WA), and by their use in research applications. The latter include comparative toxicity studies of ciguatoxins, brevetoxins, and synthetic fragments (6), and variations of cell assays based on reporter genes (7), or changes in membrane potential (8). In regulatory laboratories, cell assays for ciguatoxins are a rapid and sensitive screening tool for suspected ciguatoxic fish, especially when combined with qualitative confirmation by liquid chromatography/mass spectrometry (9).

Cell-based bioassays for the saxitoxins offer much higher sensitivity than the regulatory mouse bioassay (10). However, in contrast to the cell assays for ciguatoxins and brevetoxins, these assays are slow, requiring 24–48 h (1, 2, 11–13), and this has limited their acceptance. Sodium channel-blocking toxins can antagonize ciguatoxin and brevetoxin activity in numerous model systems, including squid axon action potentials (14), inotropic atria response (15, 16), and $^{22}Na^{+}$ influx in synaptosomes and neuroblastoma cells (17, 18). We reasoned, therefore, that the antagonism effect could be applied in cell assay format to develop a more rapid in vitro paralytic shellfish poison (PSP) detection procedure.

In a preliminary study, we demonstrated the dose-dependent detection of purified saxitoxin (STX) through antagonism of brevetoxin cytotoxicity (3). As predicted, sodium channel-blocking activity could be detected within 4–7 h in marked contrast to the prolonged incubation periods of 24–48 h required of other in vitro methods. In the present study, we extended and broadened these observations in the development of an antagonism assay format. Human cells have now been used as targets in addition to the usual murine cells, and the new antagonism format has been applied to the rapid detection and quantitation of naturally incurred PSP residues in shellfish. Antagonism assay results are compared with those determined by mouse bioassay.

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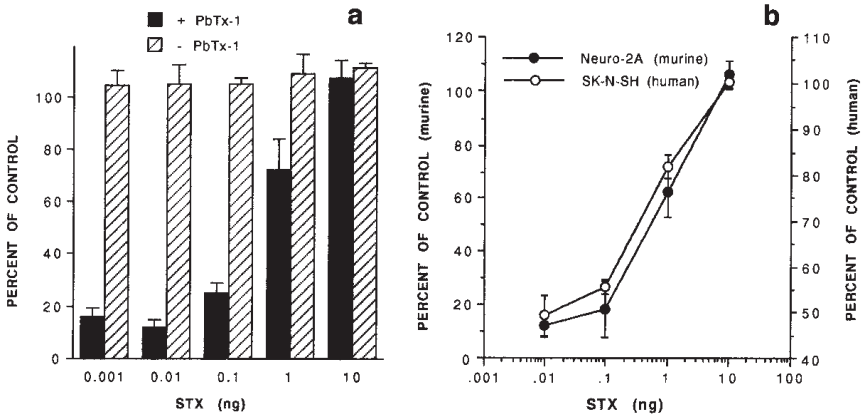

Figure 1. Detection of saxitoxin by PbTx-1 antagonism (7 h): (a) assay response of mouse Neuro-2a cells to increasing concentration (ng/well) of saxitoxin with and without PbTx-1 (6 ng/well); (b) comparative response of mouse (Neuro-2a) and human (SK-N-SH) neuroblastoma cells to saxitoxin by antagonism of PbTx-1.

Experimental

Cell Culture

Cell culture was performed as previously described (1–3) with modifications as described below. Mouse neuroblastoma cells (Neuro-2a, ATCC CCL-131) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 50 μg/mL streptomycin, and 50 units/mL penicillin. Human neuroblastoma cells (SK-N-SH, ATCC HTB-11) were grown in Eagle's Minimum Essential Medium with nonessential amino acids and Earle's Balanced Salt Solution, and supplements as described above. Cultures were maintained at 37°C in a humidified air–CO_2 atmosphere (95 + 5). The fetal bovine serum was reduced from 10 to 5% in both media formulations for cell bioassay as described below.

Shellfish Extracts and Purified Toxins

Acid extracts of butter clams (*Saxidomas giganteus*) were processed and tested by mouse bioassay (10), and then stored at –20°C for later analysis by cell bioassay, provided by the Washington State Department of Health (Seattle, WA). Purified saxitoxin stock (Calbiochem, La Jolla, CA) in 100mM AcOH was maintained at –20°C. Stock solutions of purified brevetoxin PbTx-1 (Calbiochem) and ciguatoxin CTX3C, provided by the Japan Food Research Laboratories (Tokyo, Japan), were prepared in methanol and stored at –20°C until use, as described previously (2).

Cell Bioassay

Culture plates for cell bioassay were prepared as described previously (1) with minor modifications noted below. Cell suspensions were plated into microtiter 96-well culture plates using 200 μL per well of either Neuro-2a cells at 5×10^5 cells/mL or SK-N-SH cells at ca 2.5×10^5 cells/mL. Cultures were then incubated for ca 24 h, followed by additions to test wells of 10 μL each of 1mM veratridine (Sigma Chemical Co., St. Louis, MO) and 10mM ouabain (Sigma) for Neuro-2a cells or 10 μL of 1mM veratridine without ouabain for SK-N-SH cells. Test wells then received 10 μL 0.69nM brevetoxin PbTx-1 (6 ng/well) or 10 μL 0.78 pM CTX3C (8 pg/well). Dilutions of saxitoxin standard and shellfish acid extracts were prepared and tested in replicates of 4 wells, 10 μL addition of sample per well, in the presence or absence of PbTx-1. Typically, 15 wells, containing PbTx-1/veratridine/ouabain and without PSP extract or saxitoxin, were processed as reference controls. Following all additions, plates were incubated at 37°C for 6–8 h, and then processed with MTT (Sigma) and read on a multiwell scanning spectrophotometer as previously described (1).

Results and Discussion

Our previous studies described a method for relatively rapid detection of purified saxitoxin in vitro through antagonism of brevetoxin-induced cytotoxicity in mouse neuroblastoma cells (3). In the present study, we examined the response to purified saxitoxin with both human and murine neuroblastoma cells by antagonism assay, and investigated the application of this method for detection of naturally incurred PSP residues in shellfish.

The murine cell line, Neuro 2a, previously responded optimally with a concentration of PbTx-1 at 6 ng/ well (3) and was therefore used in the present studies. Figure 1a demonstrates the dose-dependent antagonistic effect of purified saxitoxin on brevetoxin-induced mouse Neuro-2a cytotoxicity at 7 h. Effective detection ranged from 0.1 to 10 ng/well. Saxitoxin, in the absence of brevetoxin, had no effect on cell viability in the concentration range and in the relatively short incubation period tested.

Human neuroblastoma cells, SK-N-SH, were similarly applied for the detection of purified saxitoxin (Figure 1b). Unlike the mouse Neuro-2a cells, brevetoxin-induced cytotoxicity in SK-N-SH cells required only veratridine without the presence of ouabain. Although the baseline level of brevetoxin-induced cytotoxicity was less in the human cell

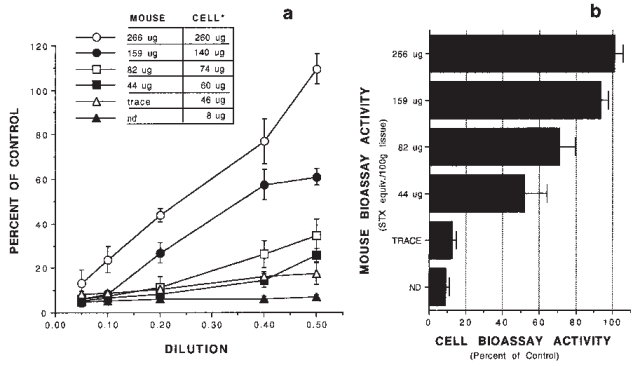

Figure 2. Antagonism assay of naturally incurred PSP in mouse neuroblastoma cells (7 h): (a) butter clam extracts tested by antagonism with PbTx-1 and compared with mouse bioassay potency (inset table); (b) butter clam extracts analyzed by antagonism of CTX3C (8 pg/well).

line than that observed with the Neuro-2a cells (data not shown), a similar saxitoxin detection limit of approximately 0.1 ng with a near linear response to 10 ng was observed in both the human and mouse neuroblastoma test methods (Figure 1b).

Antagonism assay detection of naturally incurred PSP was tested with a panel of butter clam (*S. giganteus*) extracts. Dilution-dependent dose responses were observed with these extracts by cell assay, which ranked with their relative PSP mouse bioassay potencies (Figure 2a). Based on interpolation of the 0.5 dilution extract responses and an STX positive control, calculated saxitoxin equivalents by antagonism assay demonstrated reasonable correlation with mouse bioassay potencies (Figure 2a). The slight but acceptable differences in calculated saxitoxin equivalents probably relate to the limits of precision of both the mouse (10) and cell bioassays (1). The enhanced sensitivity of the cell assay, approximately 2 μg STX equivalents per 100 g tissue compared with the mouse bioassay limit of approximately 40 μg STX equivalents (1, 10), revealed low-level PSP activity in 2 extracts that contained either trace or no detectable activity by conventional animal testing.

The mechanism of detection in the present cell assay is through antagonism of the effect of a site 5 activating neurotoxin (brevetoxin) by a site 1 blocking toxin (PSP sample), in conjunction with the potentiating effects of veratridine/ouabain. The availability of an alternative site 5 neurotoxin, ciguatoxin CTX3C, allowed additional confirmation of this mechanism. Purified ciguatoxin CTX3C was substituted for brevetoxin PbTx-1 in the antagonism assay; however, because of the enhanced potency of CTX3C (2, 3), considerably less toxin was required. The analysis of the butter clam shellfish extracts by CTX3C antagonism assay produced a relative potency ranking that was very similar to PbTx-1 antagonism results (Figures 2a and b).

The use of the antagonism assay for analysis of other molluscan species was examined with acid extracts prepared from little neck clams (*Protothaca staminea*), pink scallops (*Chlamys rubida*), blue mussels (*Mytilus edulis*), and oysters (*Crassostrea gigas*). Although both little neck clam and pink scallop extracts generally ranked in potency in a manner consistent with values determined by mouse bioassay, extracts from blue mussels and oysters produced significant interference with the antagonism assay (data not shown). These preliminary results, although limited to 3 or 4 species, suggest that there may be species-dependent interferences with the present antagonism cell assay format, and that extracts from some of the molluscan species may require additional processing before assay. Interfering factors in shellfish extracts have been noted and successfully removed in previously reported cell-based bioassays for PSP activity (12).

A preferred regulatory monitoring method would allow detection of violative samples with minimal preparation and data reduction. To accommodate these requirements, we devised a modified format of the antagonism assay that would allow simple visual assessment for identification of samples at or above regulatory action levels. The criteria were simply to identify alert level samples and not to obtain an absolute value of potency. Figure 3 shows the results obtained with this simplified format using the butter clam shellfish extracts described above (Figure 2) with a 6 h incubation. All extracts were tested at a single dilution (0.5) in replicates of 4, and compared with 8 wells tested with purified saxitoxin equivalent to an extract (diluted 0.5) at the regulatory action level of 80 μg STX/100 g shellfish. End point MTT development time was increased to allow near saturation of the saxitoxin reference control wells (approximately 45 min). With these conditions, extracts assessed by mouse bioassay to be at or above the regulatory action were clearly violative by visual assessment. Extracts found by mouse bioassay to be below the regulatory alert level were also significantly less reactive than the action level control wells in the visual antagonism cell assay. The method clearly demonstrates its utility for assessing a pass/fail value to consumable shellfish stock.

The PSP antagonism cell bioassay offers a number of advantages compared with previously described cell-based methods (1–3, 11–13). A principal benefit is the reduction of

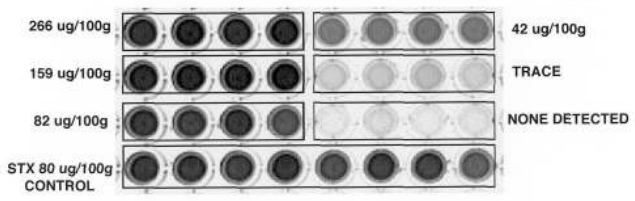

Figure 3. Visual assessment antagonism assay for PSP in butter clam extracts (6 h). Potency values represent bioactivity by mouse bioassay for tissue extracts, and equivalent regulatory action level (80 μg/100 g tissue) for purified toxin control.

incubation from 24–48 h to approximately 6 h. Although still not as rapid as the mouse bioassay (10), the antagonism assay would allow sample processing and testing within a conventional workday, thus lending more toward potential regulatory time constraints than previous in vitro methodologies. Difficulties occasionally observed in vitro with long incubation periods, such as cell overgrowth in untreated wells and accentuated cell death attributed to ouabain and veratridine, are minimized. This improves method ruggedness. These preliminary results offer the basis for continued development and application of an enhanced regulatory PSP screening method that could serve as an alternative or complementary procedure to present conventional animal testing.

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