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Pharmacological differentiation of the $P2X_7$ receptor and the maitotoxin-activated cationic channel☆

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Abstract

The ATP-$P2X_7$ receptor subtype and a maitotoxin-activated ion channel were studied to determine factors which identify them as separate entities in the control of a cytotolytic pore. Activation of ATP-$P2X_7$ receptors with 2′-3′-*O*-(benzylbenzyl) ATP (BzATP) or maitotoxin ion channels resulted in influx of ethidium bromide and cell death. Maitotoxin (25–250 pM)-induced ethidium bromide uptake and cell death was sensitive to extracellular Ca^{2+}, the ionic composition of the buffer, reduced by the calmodulin inhibitor W7, (*N*-(*s*-aminohexyl)-5-chloro-1-naphthalenesulfonamide) (10–100 μM) but unaffected by the ATP-$P2X_7$ receptor antagonist oxidized ATP, (adenosine 5′-triphosphate periodate oxidized sodium salt) (oATP). BzATP (10–200 μM)-induced ethidium bromide uptake and cell death were inhibited by oATP, unaffected by W7, inhibited by high ionic concentrations but only slightly dependant on external Ca^{2+}. These results are consistent with the existence of a pharmacological mechanism for controlling cell death consisting of an ATP-$P2X_7$ receptor, a maitotoxin-activated ion channel and a cytolytic pore.

Keywords: ATP-$P2X_7$ receptor; Maitotoxin; Cytolytic pore; Cell death; Ca^{2+}

1. Introduction

There are two major processes that have been identified by which cells are induced to die. Apoptosis or programmed cell death is an orderly process of biochemical and morphological events employed by organisms to eliminate non-functional or damaged cells. The second mode of cell death, known as necrosis, follows quite different pathways to effect cell death, a process accompanied by an inflammatory response. Although a great deal of research has been carried out characterizing how cells die using these pathways, the particular mechanisms by which cells initiate or select a particular route of cell death appear to be poorly understood.

Cell death may be initiated through a variety of signaling mechanisms including the activation of external membrane bound receptors and/or ionic pores. The role of ionic pores in the initiation of cell death has been well studied, particularly those pores which regulate intracellular Ca^{2+} levels (rev. see Kass and Orrenius, 1999). For example, maitotoxin, the most potent marine toxin yet described, has been reported to cause a variety of physiological events such as necrosis as the result of its ability to cause an increase in intracellular Ca^{2+} concentrations (Taglialatela et al., 1990; Kutty et al., 1988; Daly et al., 1995; Estacion and Schilling, 2001; Escobar et al., 1998). Initiation of these events occurs following activation of a membrane cation channel which has been suggested to be a maitotoxin receptor (Morales-Tlalpan and Vaca, 2002; Martinez-Francois et al., 2002), although no definitive proof of the presence of a maitotoxin receptor exists at the present time.

Clear examples of cell death initiated by receptor activation have also been well documented following exposure to ATP and its analogues, particularly 2′-3′-*O*-(benzylbenzyl) ATP (BzATP). These nucleotides initiate

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and promote both apoptosis and/or osmotic lysis (necrosis) in a very wide variety of cell types (Dubyak and El-Moatassim, 1993; Di Virgilio, 1995; Schulze-Lohoff et al., 1998) through the activation of distinct cell surface ATP receptors (Di Virgilio, 1995; Burnstock, 1999). Some ATP receptors, notably in the ATP-P2X sub-family, are ionotropic channels that act for example, to transport Ca^{2+} through the cell membrane (Ralevic and Burnstock, 1998) resulting in a variety of vital physiological effector responses including muscle contraction and nerve transmission (Ralevic and Burnstock, 1999; Deuchars et al., 2001; Lundy et al., 2002). The ATP-$P2X_1$ receptor (Valera et al., 1994; Brake et al., 1994) and ATP-$P2X_7$ receptor (Di Virgilio, 1995; Schulze-Lohoff et al., 1998) sub-types of ATP-P2X receptors have been implicated in the mediation of ATP-induced cell death and may in fact play a wider role in cell death initiated by other toxic insults as well. Activation of the ATP-$P2X_7$ receptor initially leads to the opening of an ionic channel, that in turn results in the secondary opening of a membrane pore. This pore in its open state, permeabilizes the membrane to a variety of larger molecular species (up to about 900 Da, Wiley et al., 1996) leading to a cascade of events that eventually results in either apoptotic or necrotic cell death (Di Virgilio, 1995; Zheng et al., 1991; Coutinho-Silva et al., 1999; Schulze-Lohoff et al., 1998; Humphreys and Dubyak, 1996; Humphreys et al., 2000).

Recent studies have suggested that there is a common link between activation of the maitotoxin receptor (ion channel) and activation of the ATP-$P2X_7$ receptor subtype which involves the secondary dilation of a pore common to both sites (Alzola et al., 2001; Schilling et al., 1999a,b). The results presented in this report provide critical pharmacological identification of two distinct sites with no apparent pharmacological interaction with one another. Furthermore, the data clearly defines a variety of factors that modulate the activity of either site and provides evidence for the direct association between the activation of either site and the subsequent dilation of what appears to be a common pore (Alzola et al., 2001; Schilling et al., 1999a,b) leading directly to the initiation of cell death.

2. Materials and methods

2.1. Chinese hamster ovary cells (K1 strain) culture

Seed cultures were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in 10% fetal calf serum in F-12 culture medium supplemented with streptomycin (100 μg/ml) and penicillin (100 IU/ml) and the medium was changed as required. Stock cultures were closely monitored and not allowed to grow to confluency prior to subculture. Test cultures were seeded so that cells were used just prior to, or at confluency.

2.2. Chemical treatment and cytotoxicity studies

For the assessment of BzATP toxicity, Chinese hamster ovary cells (K1 strain) were treated with the nucleotide dissolved in defined sucrose buffer for 1 h. The buffer was

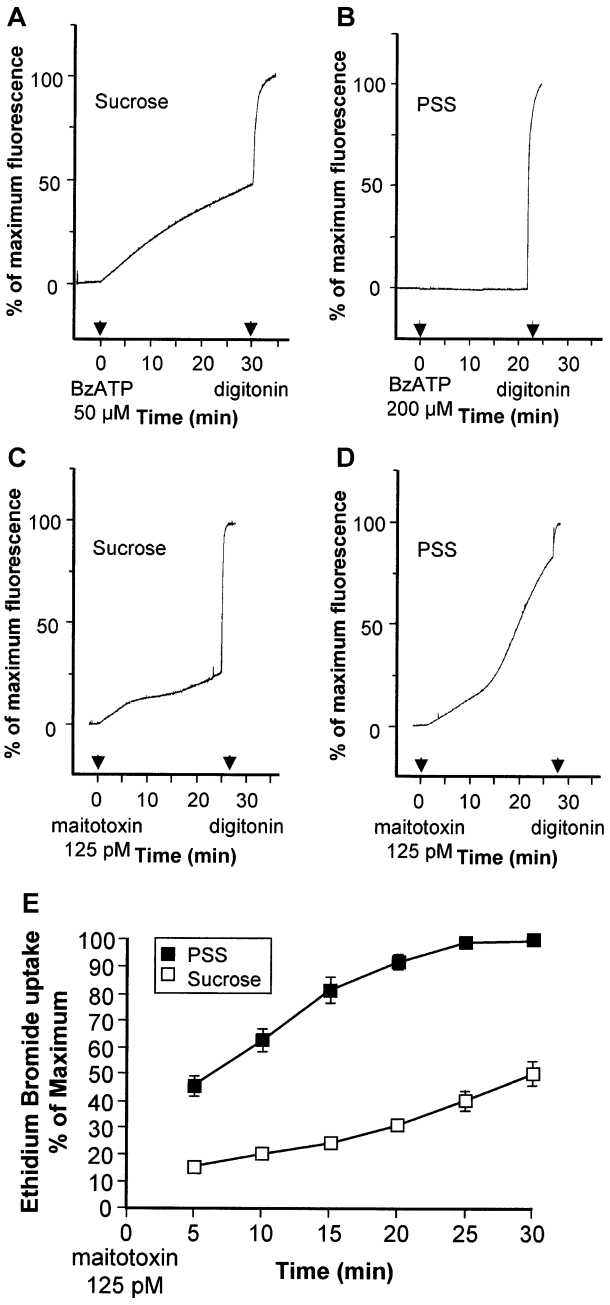

Fig. 1. Effect of the ionic concentration on maitotoxin- and BzATP-induced ethidium bromide uptake in Chinese hamster ovary cells (K1 strain). Cells were suspended in either sucrose buffer (A) or a buffer (B) containing physiological concentrations of ions (PSS) and ethidium bromide. BzATP (50 μM) induced ethidium bromide uptake much more potently in cells suspended in the non-ionic sucrose buffer (A vs. B). Maitotoxin-induced ethidium bromide uptake was evident but reduced in sucrose buffer compared with the physiological buffer (C vs. D). (E) represents a summary of ethidium uptake measured following exposure of the cells to maitotoxin in either sucrose or PSS buffer. Points represent mean ± S.E.M. ($n = 4$).

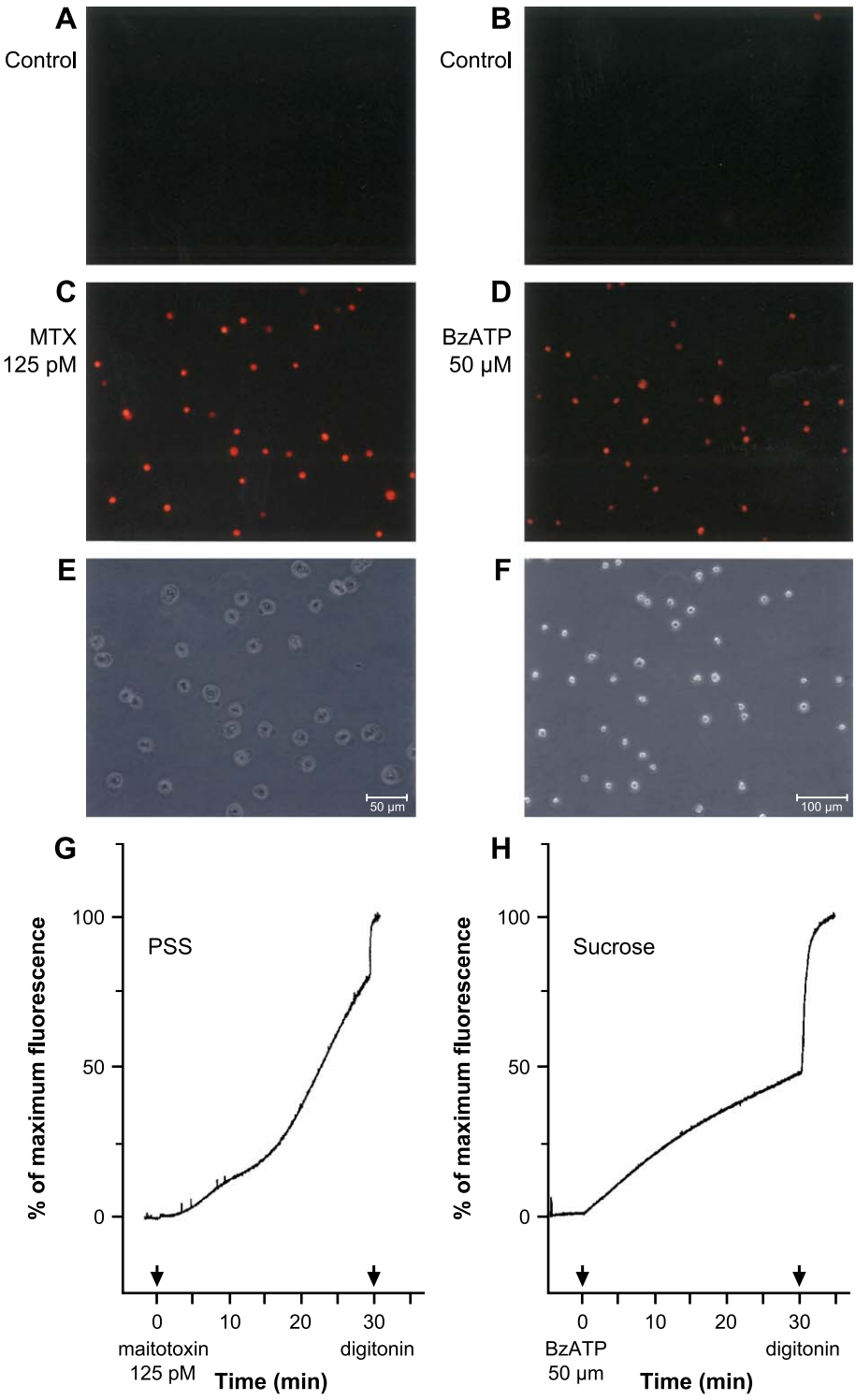

Fig. 2. Effect of the ionic concentrations of buffers on maitotoxin- and BzATP-induced ethidium bromide uptake in Chinese hamster ovary cells (K1 strain). Cells were incubated in the optimal buffer for demonstrating the activity of the agonists, maitotoxin (physiological buffer—C) and BzATP (sucrose—D). Maitotoxin (MTX 125 pM) induced dye uptake was measured by fluorescence microscopy following 30 min of exposure (C) or was followed for 30 min by kinetic analysis in physiological buffer (PSS)(G). Cells were exposed to BzATP (50 μM) and ethidium bromide uptake was measured by fluorescence microscopy at 30 min (D) or kinetically (H). Panels A and B show the lack of dye uptake in cells not exposed to either agonist, while panels E and F depict light micrographs of the samples shown in C and D, respectively ($n = 4$).

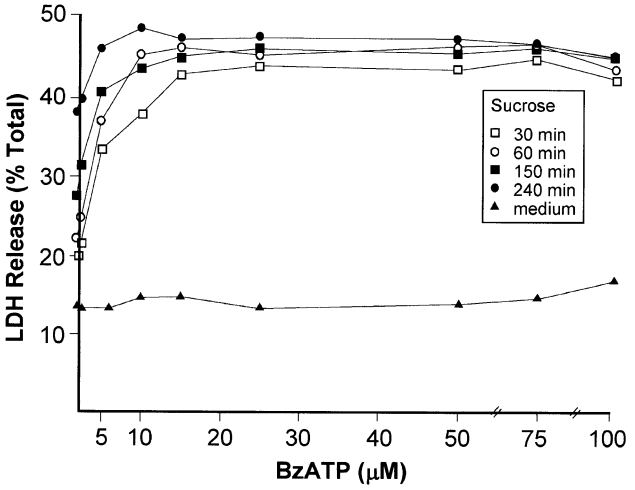

Fig. 3. BzATP-induced cytotoxicity in Chinese hamster ovary cells (K1 strain). Cells were incubated in sucrose buffer for 1 h with BzATP (5–100 μM) then refed with medium and toxicity measured 23 h later. The 24 h toxicity was determined using lactic dehydrogenase (LDH) release as the endpoint. The results are the mean of three separate experiments.

then removed and replaced with culture medium for the remaining 23 h. Maitotoxin stock solutions were made up in methanol and all final maitotoxin treatment concentrations in cell culture were achieved at 0.1% methanol. W7 (*N*-(*s*-aminohexyl)-5-chloro-1-naphthalenesulfonamide and oATP (adenosine 5′-triphosphate periodate oxidized sodium salt) were dissolved in medium and cultures were pretreated for 1 h prior to maitotoxin or BzATP addition. 24 h viability of the cultures was assessed using either the alamarBlue assay or the release of lactic dehydrogenase. To assess cytotoxicity using alamarBlue (AccuMed International, Westlake, OH, USA) the dye was added (10%, v/v) and the cultures allowed to incubate for the last 3 h of the treatment time period. The absorbances (570–600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA, USA). Lactic dehydrogenase assays were carried out using kits purchased from Sigma. Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point.

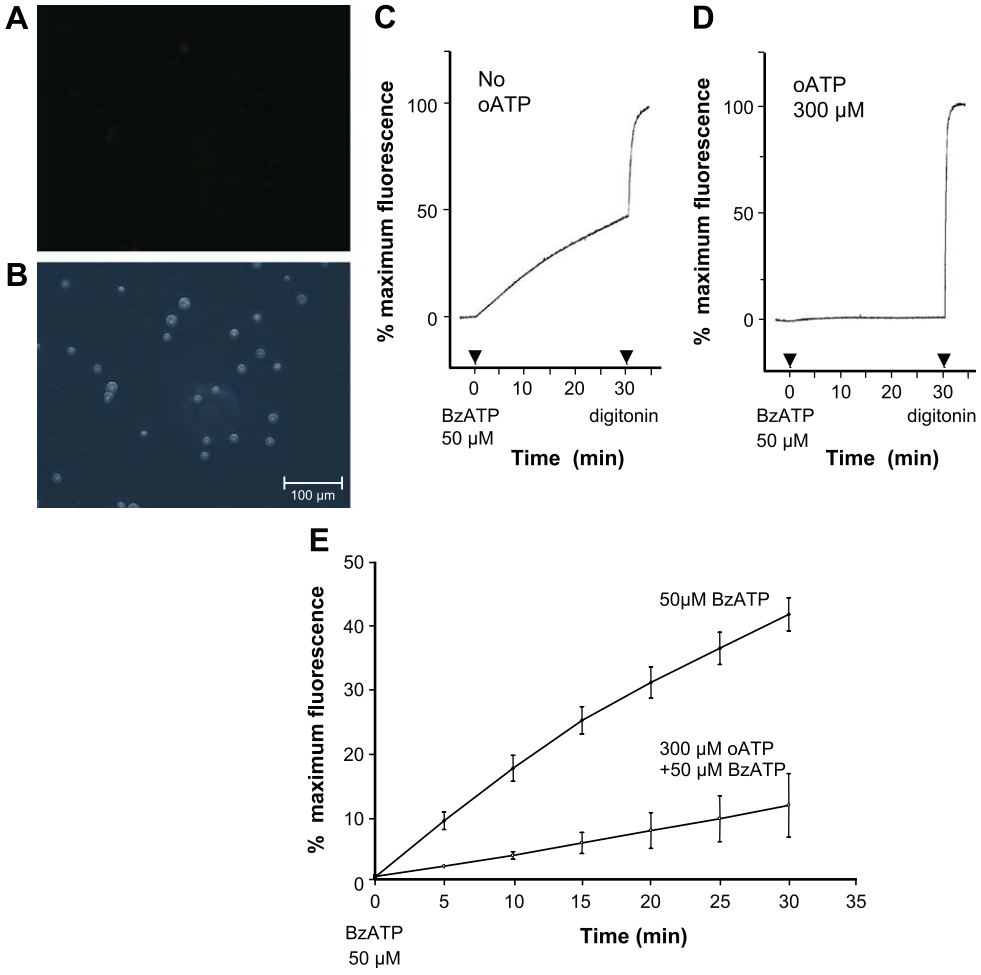

Fig. 4. Inhibitory effect of oATP on BzATP-induced ethidium bromide uptake in Chinese hamster ovary cells (K1 strain). Cells were treated with 50 μM BzATP in sucrose-based buffer containing 20 μM ethidium bromide with (Panel D)or without a 30-min preincubation with 300 μM oATP (Panel C). Samples of the cell suspension in D were removed at 30 min and examined by both light (B) and fluorescence microscopy (A), which revealed no fluorescent cells. Panel E is a summary of four experiments, each point is mean ± S.E.M.

2.3. Ethidium bromide uptake studies

Chinese hamster ovary cells (K1 strain) were allowed to grow until just confluent. The cells were gently trypsinized at room temperature and pelleted by centrifuging at $1000 \times g$ for 5 min. The pellet was suspended at a density of 10^6 cells/ml in 20 μM ethidium bromide dissolved in either physiological salt buffer (Mutini et al., 1999) or in cation deficient sucrose buffer which has been reported to optimize agonist receptor association (Michel et al., 1996). The cell suspensions were protected from light and kept on ice until used. At the time of experimentation 2 ml of cell suspension were placed into a quartz cuvette and fluorescence (excitation: 360 nm/emmision: 580 nm) was acquired for 5 min prior to drug treatment and for at least 30 min thereafter. Confirmatory evidence of ethidium bromide uptake was obtained by visualizing the cells using fluorescence microscopy. Measurements were made with reference to a maximal response induced by digitonin (10 μg/ml).

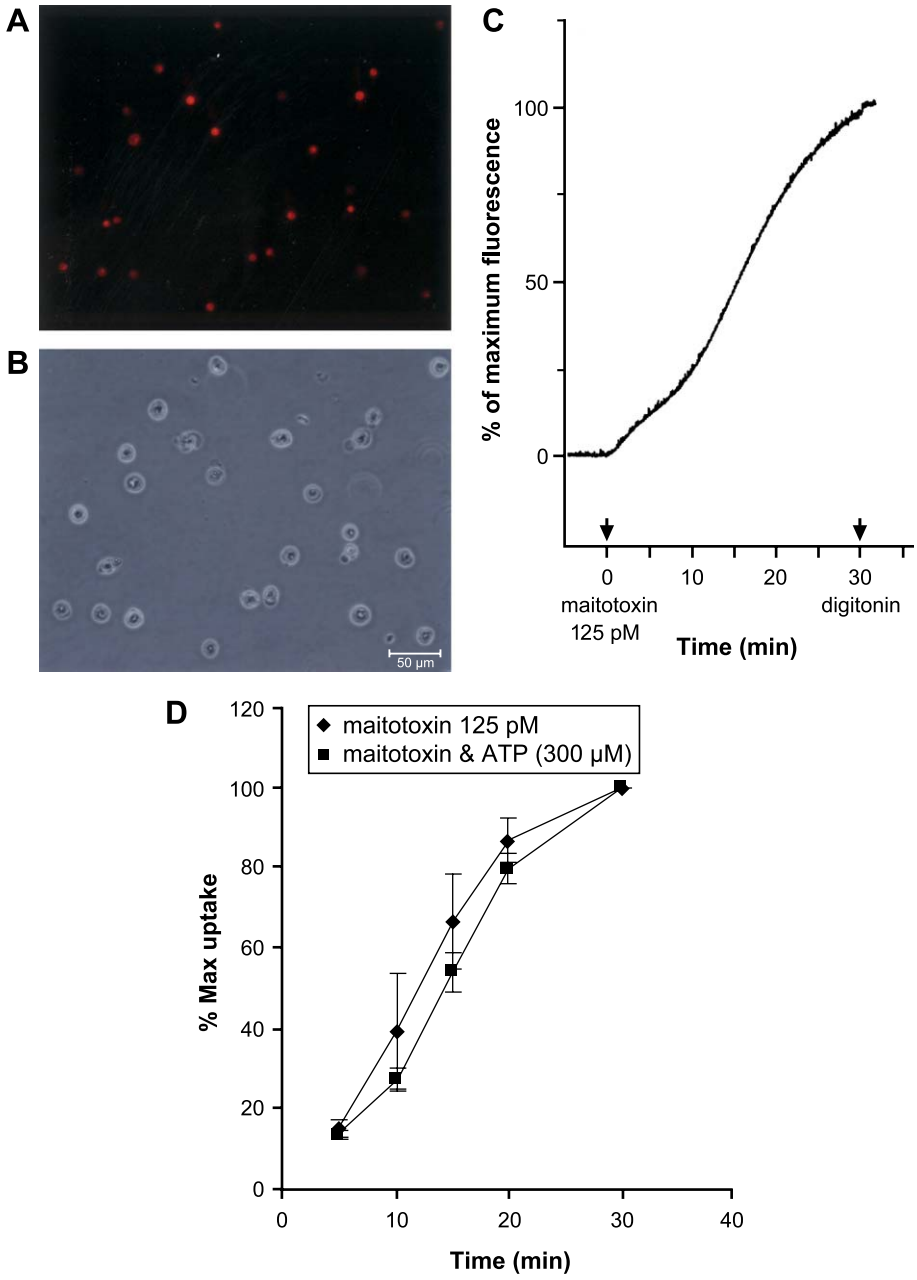

Fig. 5. Cells were incubated with 125 pM maitotoxin in physiological buffer in the presence of oATP (300 μM/30 min). Dye uptake was measured kinetically for 30 min (Panel C) at which time some cells were removed from the cuvette for analysis by fluorescent microscopy (panel A). Panel B is a light micrograph of the cells in panel A. Panel D is a summary of the effects of oATP on maitotoxin induced ethidium bromide uptake (mean ± S.E.M., $n = 4$).

2.4. Buffer solutions

Physiological salt buffer was made with the following composition in mM: NaCl, 0.5; KCl, 5; $MgSO_4$, 1; Ca^{2+} Cl_2, 1.5; Na_2HPO_4, 1; D-glucose, 5.5; $NaHCO_3$, 5 and HEPES 20 (pH 7.4). Sucrose buffer was prepared as described by Michel et al. (1996) and Surprenant et al. (1996) in order to maintain lower concentrations of interfering ions. It consisted of the following in mM: sucrose 280; Ca^{2+} Cl_2 0.5; KCl 5, D-Glucose 10, HEPES 10, *N*-methyl-D-glucamine 5, with the pH adjusted to 7.4. In studies in which the role of Ca^{2+} on ethidium bromide uptake and cell death were examined, Ca^{2+} concentrations were altered in the sucrose buffer or the physiological buffer depending on the agonist used (BzATP/sucrose; maitotoxin/physiological buffer).

2.5. Drugs

BzATP, 2′-3′-*O*-(benzylbenzyl) ATP; oATP,(adenosine 5′-triphosphate periodate oxidized sodium salt), W-7 ; (*N*-(*s*-aminohexyl)-5-chloro-1-naphthalenesulfonamide) and Ethidium bromide were obtained from Sigma (St. Louis, MO, USA) and maitotoxin was purchased from Wako (Richmond, VA, USA).

2.6. Statistical analysis

Data was analyzed by one way analysis of variance (ANOVA) followed by post hoc Neumann–Keuls testing or Dunnett's Multiple Comparison Test using Prism software (Graph Pad, San Diego, CA, USA).

3. Results

3.1. Optimal ionic requirements for maitotoxin and BzATP activity

Previous studies (i.e. Michel et al., 1996, 1999; Virginio et al., 1997; Lundy et al., 2002) suggested that the degree of activation of the ATP-$P2X_7$ receptor is heavily dependant on ionic composition of the buffer while maitotoxin has been demonstrated to be very toxic in more traditional ionic buffers. The present studies suggested that the degree of activation of the ATP-$P2X_7$ receptor or the maitotoxin-activated ionic channel was dependant on the concentration of what appeared to be critical ions in the medium and that ionic manipulation of the environment might provide a method of differentiating the two structures from each other. Studies were carried out in attempts to reveal separate membrane-activated sites based on the ionic requirements for activation.

Fig. 1 revealed the time and ionic-dependence of ethidium bromide uptake following exposure of Chinese hamster ovary cells (K1 strain) to BzATP or maitotoxin and subsequent fluorescence signals resulting from binding to DNA. When cells incubated in sucrose buffer were exposed to 50 μM BzATP, a robust and time dependent increase in ethidium bromide uptake was observed over the 30-min test period (Fig. 1A). In contrast, when the cells were incubated in buffer containing physiologically relevant ion concentrations, even a much higher concentration of BzATP (200 μM) failed to induce significant dye uptake over baseline values (Fig. 1B). Similar studies revealed that maitotoxin-induced ethidium bromide uptake was much less dependent on the ionic concentration of the test buffer than that of BzATP and that the presence of ions (in physiological buffer, Fig. 1D) enhanced maitotoxin evoked ethidium bromide uptake compared to that obtained in sucrose buffer (Fig. 1C) A comparison between the effects of maitotoxin (125 pM)-induced ethidium bromide uptake in physiological buffer (■) with its effects in sucrose buffer (□) is

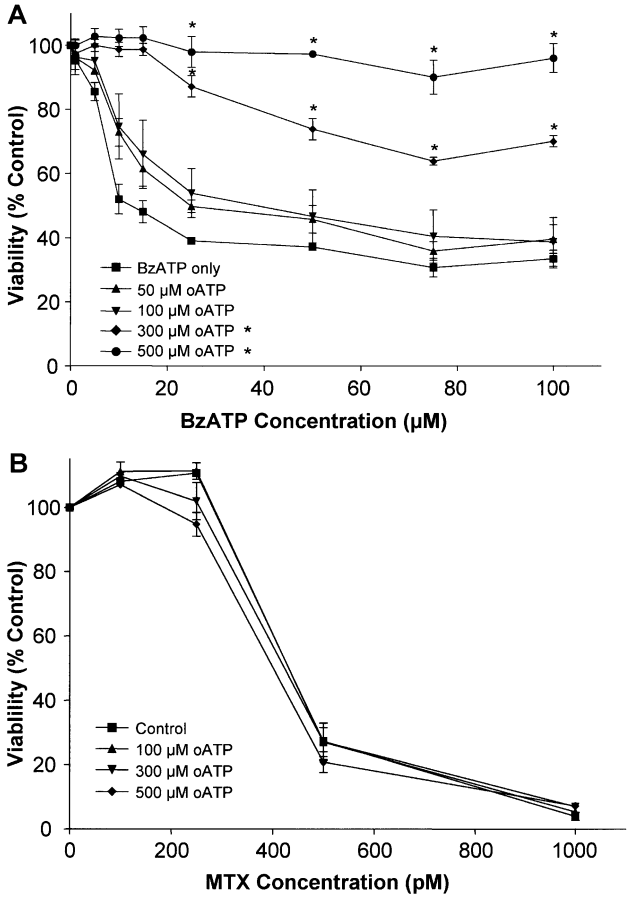

Fig. 6. Just confluent cultures were treated with various concentrations of oATP for 1 h prior to exposure to increasing concentrations of BzATP (A) or maitotoxin (MTX) (B). BzATP incubations were carried out in sucrose buffer for 1 h prior to aspiration and then refed with medium. Maitotoxin (MTX) exposures were carried out in culture medium and were not refed. The data is normalized as a percent of the toxicity of vehicle treated controls. All data represents the mean ± S.E.M obtained from three separate experiments. Asterisks in (A) denote statistically significant protection compared to cells that were not pretreated ($p < 0.05$, Dunnett's Multiple Comparison Test).

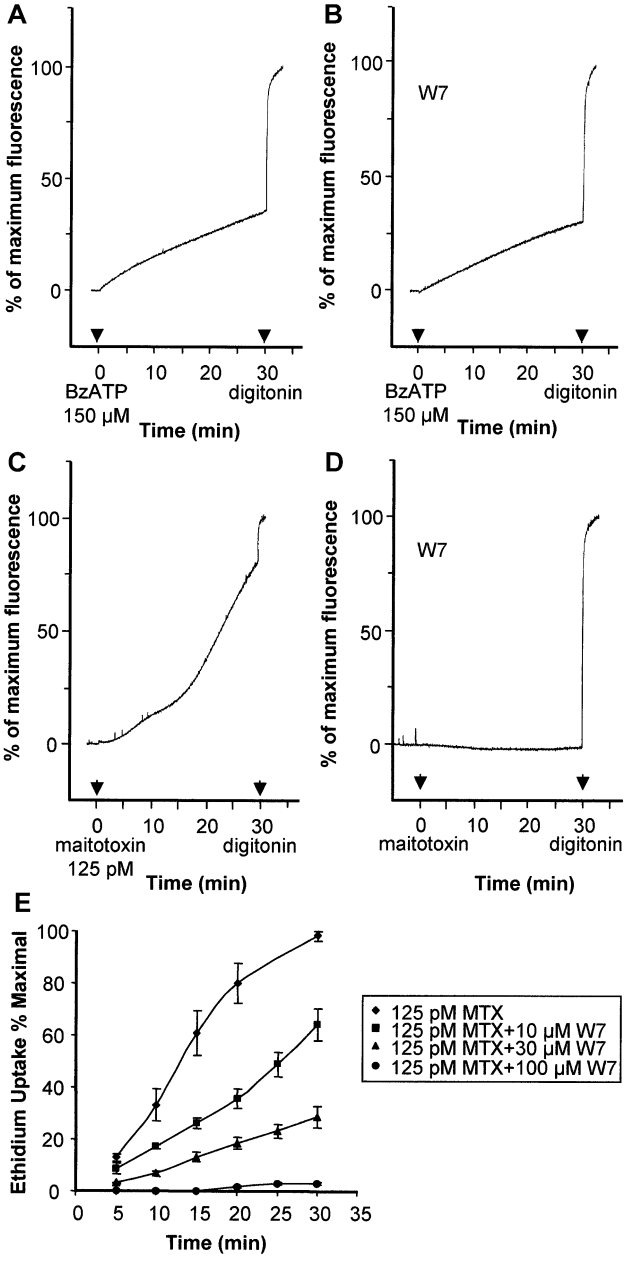

Fig. 7. Cultures were incubated in sucrose buffer and exposed to BzATP alone (150 μM; panel A) or following W7 (300 μM–30 min—panel B). Panel C depicts a typical result showing ethidium bromide uptake following the incubation of 125 pM maitotoxin alone in physiological buffer and panel D shows the inhibition of ethidium bromide uptake following preincubation with W7 (300 μM–30 min). Panel E shows the dose-dependent inhibitory properties of W7 on ethidium bromide uptake following maitotoxin (MTX) (each point is mean ± S.E.M., $n=4$).

supplied in Fig. 1E. Further confirmation of these findings was obtained using fluorescence microscopy (Fig. 2) to visualize the effects of maitotoxin (125 pM) and BzATP (50 μM) on Chinese hamster ovary cells (K1 strain) under the two respective ionic conditions (maitotoxin in physiological buffer, and BzATP in sucrose). Few or no fluorescent cells were noted following vehicle treatment after a 30-min incubation (Fig. 2A,B). However, both maitotoxin (Fig. 2G) and BzATP (Fig. 2H) treatment caused a time dependent uptake of ethidium bromide when measured kinetically in appropriate buffers. Samples of these cell suspensions were taken from the cuvette and photographed at the end of the 30-min incubation period with either agonist. Most of the cells that could be seen by light microscopy (Fig. 2E,F) demonstrated fluorescence indicative of ethidium bromide uptake (Fig. 2C,D).

Studies carried out to determine the toxicity of BzATP in Chinese hamster ovary cells (K1 strain) cultures also showed the dependence of the lethal actions of BzATP on the ionic concentration of the buffer/medium bathing the cells. When the cultures were exposed to BzATP in Ham's F-12 culture medium (~ 130 mM NaCl), no toxicity could be detected even at 100 μM concentrations indicated by lactic dehydrogenase release as a measure of toxicity. In contrast, in sucrose buffer (Fig. 3), BzATP induced near

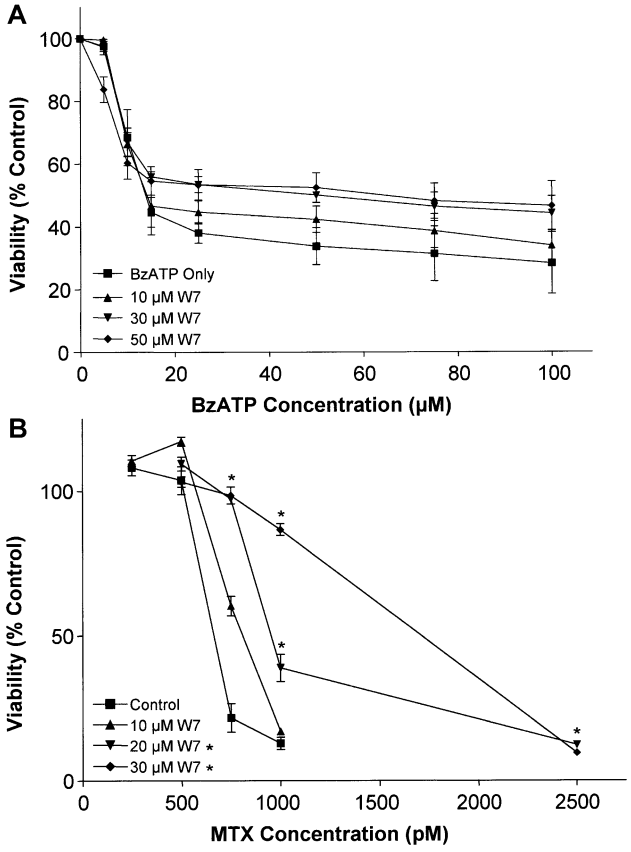

Fig. 8. Effect of W7 on BzATP- (panel A) and maitotoxin (MTX)- (panel B) induced cytotoxicity measured by AlamarBlue assay at 24 h, in Chinese hamster ovary cells (K1 strain). Just confluent cultures were treated with various concentrations of W7 1 h prior to exposure to increasing concentrations of BzATP (A) or maitotoxin (B) in appropriate buffers. W7 and maitotoxin incubations were carried out in culture medium with no subsequent medium change. The data is normalized as a percent of the toxicity of vehicle treated controls. All data points represents the mean ± S.E.M. ($n=3$). Asterisks denote statistically significant protection compared to no pretreatment ($p<0.05$, Dunnett's Multiple Comparison Test).

maximal 24 h toxicity (which was only about 50% of the total cells) following 30 min exposure to BzATP prior to aspiration of the sucrose and replacement with Ham's F-12 culture medium. BzATP also induced a similar toxic response in sucrose buffer as assessed using the alamarBlue viability assay (see Fig. 6A). Subsequent to the study presented in Fig. 3 all assays of cytotoxicity were carried out using the alamarBlue assay.

3.2. The effect of antagonists on maitotoxin and BzATP-induced ethidium bromide uptake and toxicity

The lack of interaction between the effects of maitotoxin and BzATP was further characterized by measuring the effects of agonist/antagonist activity as reflected by pore dilation following exposure to either maitotoxin or BzATP, utilizing both ethidium bromide uptake and cytotoxicity as endpoints (Figs. 4, 5 and 6). Incubation of the cells with the selective ATP-P2X$_7$ receptor antagonist oATP 30 min prior to BzATP exposure (in sucrose buffer) totally inhibited ethidium bromide uptake over the 30 min incubation period (Fig. 4C vs. D). Fig. 4E shows ethidium bromide uptake (mean ± S.E.M.) in cells exposed to BzATP in the presence and absence of the ATP-P2X$_7$ receptor blocker oATP. Cells exposed to vehicle exhibited no measurable staining by fluorescence microscopy (Fig. 4A) as compared to the BzATP-induced stained cells previously shown in Fig. 2D. Fig. 4E shows a summary of the effect of oATP on ethidium bromide uptake following BzATP exposure (each point = mean ± S.E.M.). In contrast, the results in Fig. 5 showed that pretreatment of cells with a concentration of oATP (300 μM), sufficient to block the effects of BzATP, had no significant inhibitory effect on the ethidium bromide uptake in Chinese hamster ovary cells (K1 strain) treated with 125 pM maitotoxin (in physiological buffer) as assessed either microscopically (Fig. 5A) or kinetically (Fig. 5D). The points in Fig. 5D represents the mean ± S.E.M. of the uptake obtained following maitotoxin alone as opposed to that obtained following preincubation with 300 μM oATP. Similar findings were also obtained when the effects of oATP on BzATP-induced toxicity were examined (Fig. 6A) contrasted with the lack of effect on maitotoxin-induced toxicity (Fig. 6B). When Chinese hamster ovary cells (K1 strain) were incubated with BzATP in sucrose buffer for 1 h, the metabolic viability of the cells at 24 h (as measured by alamarBlue) declined in a concentration dependent fashion

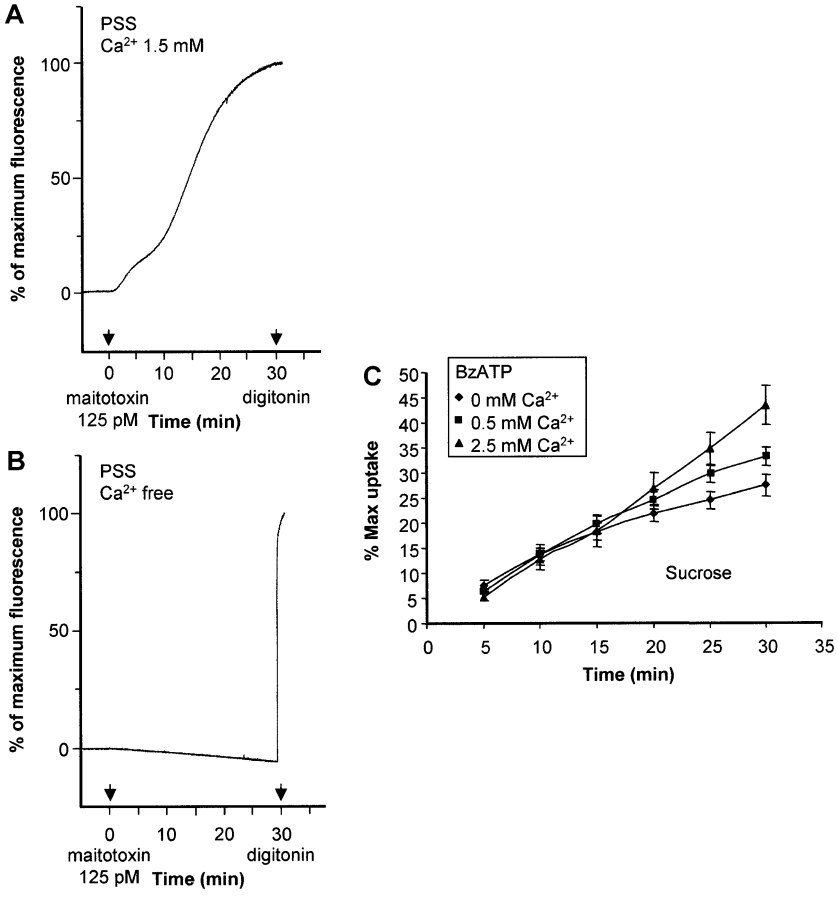

Fig. 9. Cells incubated in physiological buffer (PSS) containing 1.5 mM Ca^{2+} and exposed to 125 pM maitotoxin exhibited maximal increases in fluorescence (A), while cells bathed in Ca^{2+} free buffer showed no response to the toxin (B). In contrast, Chinese hamster ovary cells (K1 strain) suspended in sucrose buffer and exposed to BzATP exhibited no statistically significant dependence on Ca^{2+} concentration (C, mean ± S.E.M., $n = 3$).

to less then 40% of control values. However, preincubation of the cultures with increasing concentrations of oATP prior to BzATP treatment, led to a concentration related protection from cell death which was complete at 500 μM oATP (Fig. 6A). Studies were then carried out in order to examine the possible role of the calmodulin inhibitor W7 on the activation of the ATP-P2X$_7$ receptor site with respect to the uptake of ethidium bromide. Exposure of cells to BzATP (150 μM) resulted in ethidium bromide uptake (Fig. 7A) a response which was unaffected by prior incubation for 30 min with 300 μM W7 (Fig. 7B). In contrast, the rapid uptake of ethidium bromide induced by 125 pM maitotoxin was totally abolished by a 15-min preincubation with this concentration of W7 (Fig. 7C vs. D). Fig. 7E represents a summary of the inhibition of maitotoxin-induced ethidium bromide uptake in the presence of increasing concentrations of W7 (each point = mean ± S.E.M., $n = 4$). Experiments were then carried out to examine the role of W7 on the cytotoxicity induced by BzATP or maitotoxin. W7 exerted differential and selective effects on the cytotoxicity induced by maitotoxin and BzATP similar to those it caused on ethidium bromide uptake measurements. Preincubation of cells with W7 prior to maitotoxin exposure proved to be cytoprotective in a concentration dependent fashion (Fig. 8B), but was only marginally effective in preventing BzATP toxicity (Fig. 8A).

3.3. Effect of external Ca^{2+} concentration on maitotoxin- and BzATP-induced ethidium bromide uptake and toxicity

Studies were then carried out to examine the role of Ca^{2+} with respect to the activation of the two proposed receptors in order to determine whether differences existed in the Ca^{2+} requirement for receptor activation.

The results show a marked dependence on external Ca^{2+} of maitotoxin-induced ethidium bromide uptake in Chinese hamster ovary cells (K1 strain) (Fig. 9A vs. B) Maitotoxin (125 pM), resulted in a steep increase in ethidium bromide uptake which was apparent when the assays were carried out in buffer containing 1.5 mM Ca^{2+} (Fig. 9A). This Ca^{2+} concentration was near optimal in facilitating ethidium bromide uptake (results not shown). However, no measurable dye uptake was apparent over 30 min when Ca^{2+} was omitted from the external medium (Fig. 9B). In additional experiments (results not shown), cells exposed to maitotoxin in Ca^{2+} free physiological buffer failed to take up ethidium bromide until Ca^{2+} (1.5 mM) was added to the cuvette. This invariably prompted an immediate increase in the uptake of ethidium bromide that ultimately reached maximal levels. BzATP, unlike maitotoxin, induced ethidium bromide uptake (Fig. 9C) in the absence of any added Ca^{2+} to the sucrose buffer. Moreover, analysis of the three curves depicted in this figure failed to reveal any statistically significant effect of external Ca^{2+} concentration on ethidium bromide uptake (ANOVA). However, the shape of the ethidium bromide uptake curves (Fig. 9C) suggests some divergence in the ethidium bromide uptake at time periods longer than 20 min following agonist exposure. Initial experiments have confirmed that Ca^{2+} enhanced ethidium bromide uptake during incubation periods longer than the 30-min periods examined here. This suggests the possibility of an increasing effect of external Ca^{2+} with time and may correlate with the initiation of the second phase of the biphasic response observed following exposure to BzATP.

Fig. 10 shows the toxicity expressed in cells exposed to either BzATP (Fig. 10A) or maitotoxin (MTX) (Fig. 10B). BzATP toxicity was examined in sucrose buffer at various Ca^{2+} concentrations (10 μM–2.5 mM). BzATP was not toxic when the incubation was carried out in the absence of Ca^{2+}. While at low concentrations of Ca^{2+} (100 μM), a shallow decline in cell viability following BzATP became

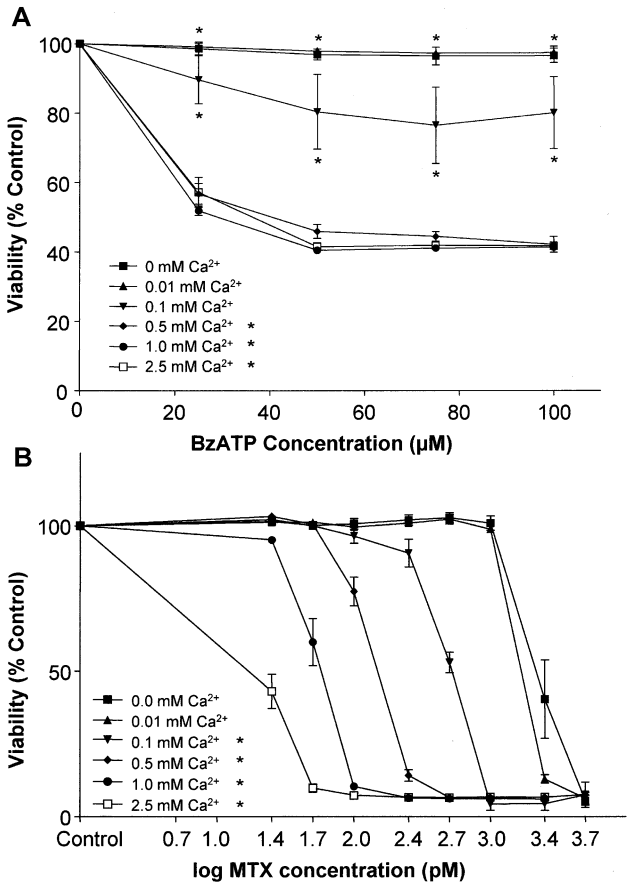

Fig. 10. Effect of external Ca^{2+} concentration on BzATP (Panel A) and maitotoxin (MTX, Panel B) induced cytotoxicity in Chinese hamster ovary cells (K1 strain). BzATP (1 h) incubations were carried out in sucrose buffer containing various Ca^{2+} concentrations. Maitotoxin incubations were carried out in medium containing increasing Ca^{2+} concentrations. Cell death was measured 24 h post-treatment using the alamarBlue cytotoxicity assay. Both BzATP (A) and maitotoxin (B) toxicity were shown to be dependent on external Ca^{2+} concentration. The data is normalized as a percent of the toxicity of vehicle treated controls. Points represent mean ± S.E.M., $n = 3$). Asterisks denote statistically significant increase in toxicity beyond that obtained in 0 Ca^{2+} ($p < 0.05$, Dunnett's Multiple Comparison Test).

evident, which was maximal but incomplete at 500 μM (60% of total cells exposed). The Ca^{2+} dependence on maitotoxin-induced cell toxicity (Fig. 10B) was different in character than that following BzATP. Although maitotoxin was still extremely toxic even in the absence of added Ca^{2+} ($LC_{50} = 2.4 \pm 0.6$ nM, $n = 3$), the addition of Ca^{2+} resulted in a profound concentration dependent increase in toxicity so that at 2.5 mM Ca^{2+}, LC_{50} values were decreased by over two log orders of magnitude ($LC_{50} = 22.4 \pm 3.7$ pM, $n = 3$). In addition, and in contrast to BzATP cytotoxicity, maitotoxin toxicity approached 100% at higher concentrations.

4. Discussion

The results of this study offer strong evidence for the existence of a separate ATP-$P2X_7$ receptor and a maitotoxin-activated cationic channel (a maitotoxin receptor?), which upon activation, independently initiated the dilation of what appears to be a single membrane pore. Pore dilation resulted in the uptake of large molecular weight substances such as ethidium bromide and subsequent cell death in Chinese hamster ovary cells (K1 strain). The existence of ATP-$P2X_7$ receptors in Chinese hamster ovary cells (K1 strain) has been previously demonstrated (Michel et al., 1998) and was confirmed by reverse transcription polymerase chain reaction on RNA from these particular cells (Lundy et al., unpublished observations).

Previously published work had demonstrated the inhibitory effects of certain ionic species (notably sodium and divalent cations) on agonist/ATP-$P2X_7$ receptor association (Michel et al., 1999; Virginio et al., 1997, 1999; Song and Chueh, 1996; Lundy et al., 2002). The current results are consistent with the requirement for a cation deficient environment for maximal BzATP-induced ATP-$P2X_7$ receptor activation, subsequent pore dilation and ultimate cell death. In contrast, maitotoxin activation of the cytolytic pore was favored following exposure of cells to maitotoxin at more "normal" physiological ionic concentrations typified by high sodium.

The activation of the two distinct sites was also dependant to different degrees on the Ca^{2+} concentration in the incubation medium. Maitotoxin-induced ionic channel activation was clearly heavily dependant on external Ca^{2+} concentrations (see Morales-Tlalpan and Vaca, 2002). It appears therefore that Ca^{2+} is critical for the initiation or propagation of maitotoxin effects. On the other hand, although Ca^{2+} greatly enhanced the toxicity of maitotoxin in a concentration related fashion, toxicity was reduced but not abolished in Ca^{2+} deficient environments. The present results suggest that extracellular Ca^{2+} may not be absolutely essential for initiation of, but rather greatly potentiates maitotoxin-induced toxicity. The lack of a direct correlation between the requirement for Ca^{2+} to induce ethidium bromide uptake as opposed to its role in cytotoxic effects may be due to the fact that ethidium bromide uptake studies were carried out over 30 min, whereas the toxicity studies were quantified after 24 h.

In contrast to the effects observed with maitotoxin, the activation of the ATP-$P2X_7$ receptor was considerably less dependant on the concentration of Ca^{2+} as judged by either ethidium bromide uptake or cytotoxicity. Lower concentration of Ca^{2+} (100 uM) appeared necessary for the expression of the lethal effects of BzATP, however lethality was not apparently dependant on Ca^{2+} in a concentration dependant fashion. Moreover, a significant degree of the BzATP-induced ethidium bromide uptake could also be demonstrated even in the absence of added Ca^{2+}.

Perhaps the most compelling argument for the existence of completely a separate ATP-$P2X_7$ receptor from the maitotoxin-activated ionic channel (or receptor—see Introduction) emerged from experiments in which different pharmacological antagonists were employed. Concentrations of oATP, a selective antagonist of the ATP-$P2X_7$ receptor site (Murgia et al., 1993) totally abolished responses to BzATP-activated ethidium bromide uptake and cytotoxicity, while it had little or no effect on these changes induced by maitotoxin. The differential inhibition of the sites activated by BzATP and maitotoxin by the calmodulin inhibitor W7 also offers a clear distinction between the ATP-P2X.$_7$ receptor and the maitotoxin activated cation channel. W7 demonstrated a clear concentration dependant inhibition of both maitotoxin-induced ethidium bromide uptake and cell death but had no significant effects on similar parameters induced by ATP-$P2X_7$ receptor stimulation induced by BzATP. Furthermore, a second calmodulin inhibitor, triflupromazine, also blocked the maitotoxin-induced rise in ethidium bromide and the toxicity of maitotoxin dose dependently (1–10 μM), an effect which was more potent than the effects resulting from W7. Although the mechanism of W7 and triflupromazine in the prevention of toxicity and the inhibition of the pore opening properties of maitotoxin is currently unclear, the fact that calmodulin inhibitors and oATP are specific for the inhibition of maitotoxin responses as opposed to BzATP responses offers additional strong evidence that the ATP-$P2X_7$ receptor and the maitotoxin active sites are separate from each other and supports previous studies in which HEK cells devoid of ATP-$P2X_7$ receptors fully responded to maitotoxin exposure (Schilling et al., 1999b).

It is interesting to speculate on the reasons that cells, including human skin fibroblasts (Schilling et al., 1999a), Chinese hamster ovary cells (K1 strain) and likely many other cell types, contain at least what appears to be two completely different receptors which are involved in the initiation of cytolysis following the opening of a membrane pore. Previous studies have shown that maitotoxin leads to necrosis in the absence of apoptotic cell death (Kutty et al., 1988; Zhao et al., 1999). In sharp contrast, stimulation of the ATP-$P2X_7$ receptor with BzATP leads to a biochemical

cascade including caspase induction, DNA fragmentation, poly (ADP-ribose)polymerase cleavage, release of cytokines, the activation of phospholipases and the induction of apoptosis which is followed in a proportion of the affected cells by secondary necrosis (Di Virgilio, 1995; Song and Chueh, 1996; Coutinho-Silva et al., 1999; Schulze-Lohoff et al., 1998; Humphreys and Dubyak, 1996; Humphreys et al., 2000). The presence of two receptors or one receptor and one cationic channel associated with at least one membrane pore (Alzola et al., 2001; Schilling et al., 1999a,b), may provide the cell with subtle methods by which to modulate its survival. Preferential activation of one of these sites may partly explain why certain insults cause cells to take a given route to cell death. Further evaluation of such speculation awaits identification of the maitotoxin site as a receptor (Martinez-Francois et al., 2002; Morales-Tlalpan and Vaca, 2002), as well as elucidation of its mechanism of action with respect to the inhibition of calmodulin.

Although the ATP-P2X$_7$ receptor in vivo is activated by ATP and presumably other pharmacological agents, it has yet to be demonstrated whether the maitotoxin-activated ionic channel responds to other endogenous or exogenous agonists resulting in cell death. It is presently also unclear whether the cytolytic pore diameter could be modified directly without primary activation of a receptor/ionic channel. However, maitotoxin and BzATP are known to have very interesting pharmacological activity in a variety of tissues related to signal transduction. For example, maitotoxin cation channels and ATP-P2X$_7$ receptor sites have both been clearly identified on nerve endings where they sub-serve Ca^{2+} entry followed by neurotransmitter release (Taglialatela et al., 1990; Deuchars et al., 2001; Lundy et al., 2002). Furthermore, it appears inherently unlikely that maitotoxin-activated channels would be resident on terrestrial mammalian tissue solely to respond to a rare marine toxin. Therefore the identification of possible endogenous substances that activate the maitotoxin site or the cytolytic pore directly would be of considerable interest. In order to identify such compounds, it will be necessary to define the maitotoxin site as a receptor and identify specific blocking agents for it, or to elucidate downstream events utilizing other pharmacological agents such as W7.

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