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Prostate cancer cells stimulated by calcium-mediated activation of protein kinase C undergo a refractory period before re-releasing calcium-bearing microvesicles

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ABSTRACT

MVs are released in response to several stress agents, in an attempt to prevent continued cellular damage. After an initial stimulus of prostate cancer cells with sublytic C5b-9 and activation of MV release through PKC, cells take at least 20 min to fully recover their ability to microvesiculate. This release of MVs through activation of sublytic C5b-9 was inhibited by the PKC inhibitor bisindoylmaleimide I but not the Rho kinase inhibitor, Y27632. After stimulus there is a rise of 79 nMs^{-1} over 11 s, reaching a peak $[Ca^{2+}]_i$ of 920 nM. The concentration of cytosolic calcium then falls steadily at 2.4 nMs^{-1} over 109 s reaching baseline levels (50–100 nM) within 10–15 min. In PC3 cells the rate of release of MVs from stimulated cells also reaches a minimum within 10–15 min. Using fura-2 AM-loaded cells, upon stimulation, cells were found to release MVs with a concentration of intravesicular calcium estimated at ~430 nM.

1. Introduction

Cells typically produce a variety of vesicles, some of which including lysosomes, endosomes, multivesicular bodies and various transport and secretory vehicles remain within the cell. Of those that are released, exosomes are distinguished from microvesicles (MVs) by their smaller size, 50–100 nm, lower expression of phosphatidylserine (PtSer) and by expression of the surface markers Alix, CD63 and TSG101 [1,2]. The established MV characteristics include size, ~100 nm μm in diameter, and for all MVs, the expression of negatively charged phospholipids such as phosphatidylserine (PtSer) and phosphotidylcholine translocated on to the outer leaflet of the plasma membrane [1] as seen during early apoptosis [3].

The formation of MVs as a characteristic feature of cells undergoing early stage apoptosis [4] is typified by PtSer externalisation and the calpain-mediated cleavage of the actin cytoskeleton due to a rise in intracellular Ca^{2+} [1]. This rise in $[Ca^{2+}]_i$ originates mostly from external sources and enters the cell through ion channels [3], membrane pores, including membrane attack complex (MAC) or through certain types of cell damage [1] and even interaction with intracellular pathogens including certain protozoan parasites [5] and viruses [6].

We report here insights into the pathway of MV release following sublytic MAC stimulation and whether cells can effectively receive repeated stimuli. We also investigate the intravesicular content of $[Ca^{2+}]_i$ in MVs and begin to look at the potential for calcium to be released from cells, speculating on any possible roles ranging from calcium homoeostasis and transport to cell protection.

2. Materials and methods

2.1. Stimulation of cells with sublytic complement (NHS) or BzATP

To stimulate MV release with sublytic complement, PC3 cells (1×10^5 cells/ml) presensitized (for 30 min/4 °C) in rabbit anti-PC3 cell membrane antiserum (5%, v/v) in pre-warmed RPMI and 2 mM $CaCl_2$ were treated with exosome-/MV-free 5% NHS (human serum type AB; Sigma) (0.22 μm filtered and spun at 100,000 *g*/16 h). Cells were also stimulated with 200 μM BzATP (37 °C/30 min) or 5 μg/ml LPS, for release of MVs, in the presence of 2 mM $CaCl_2$. Where needed cells were also treated with calcium ionophore, A23187 at 5 μM/20 min. For complement-mediated release, the

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concentrations of complement and anti-PC3 serum used were established using a checkerboard titration. Thus using 5% antibody (for 30 min at 4 °C) and 5% NHS (10 min/37 °C) less than 5% non-viable cells were obtained, which was taken as a sublytic level. Released MVs were recovered by differential centrifugation and quantified as described below. Where necessary, heat inactivation of NHS was carried out at 56 °C for 30 min. C9-depleted serum was purchased (SigmaAldrich).

2.2. Isolation of PC3-released MVs

Metastatic prostate cancer (PCa) cells, PC3 (used between passage 3 and 8) were cultured and upon stimulation MVs isolated as follows. Essentially, cells in culture medium comprising RPMI 1640 with 5% EV-free FBS rendered MV- and exosome-free (EV-free) by centrifugation at 4000 *g*, filtration using a 0.22 μm filter and centrifugation at 160,000 *g* for 90 min, were separated by centrifugation at 160 *g* for 5 min and then again twice more at 4000 *g* for 60 min. The supernatant was immediately centrifuged at 15,000 *g* for 90 min and the following supernatant either discarded, or used for exosome isolation (2x 100,000 *g* spins for 1 h), and the pellet suspended in 250 μl sterile, EV-free PBS before a final centrifugation at 15,000 *g* for 15 min. The resulting pellet, in 100 μl sterile MV-/exosome-free Dulbecco's PBS (Mg^{2+} and Ca^{2+}-free) was labelled with Annexin V (see labelling for phosphatidylserine, PtSer, below) and quantified and sized by Nanoparticle Tracking Analysis (NTA) using the NanoSight LM20 according to the manufacturer's instructions (NanoSight, U.K.).

2.3. Labelling for PtSer and electron microscopy

Detection of PtSer was carried out as described earlier [7,8] as was electron microscopy of negatively stained EVs exosomes and microvesicles (EMVs) [7,8].

2.4. Assessment of apoptosis by flow cytometry

To assess the level of apoptosis of cells releasing MVs, cells were stained with annexin V (AnV) and 7-aminoactinomycin D (7-AAD) (Guava Nexin Reagent). Cells positive for AnV only (early apoptotic) or AnV and 7-AAD-positive cells (late apoptotic) were quantified over 60 min by flow cytometry.

2.5. Restimulation of MV release

PC3 cells (1×10^6) were rested in RPMI for 30 min, then washed and stimulated with 5% EV-free NHS and 2 mM $CaCl_2$ (30 min/37 °C) in a shaking water bath. The cells were rested in warmed RPMI until required. At 0, 10, 20, 30, 60, 90 and 120 min time points, the cells were pelleted and suspended in EV-free 5% NHS and 2 mM $CaCl_2$, for 30 min/37 °C with rotation. The supernatant was then collected and MVs isolated.

2.6. Measurement of intracellular calcium

Measurements of intracellular calcium at 37 °C in PNT2 cells, in the presence or not of the calcium chelator 1,2-bis(*o*-aminophenoxy) ethane-*N, N, N′,N′*-tetra-acetic acid tetra (acetoxymethyl) ester, BAPTA-AM (0.1 μM) were made before and after addition of PC3 MVs, this calcium chelator enabling establishment of a zero calcium level within the cells for calibration purposes. After pretreating the cells in phenol red-free RPMI (Invitrogen) for 30 min and resuspension in physiological salt as described before [10], the PNT2 cells (1×10^6/ml) were loaded with 2 mM fura 2-AM with stirring and A_{505} monitored on a spectrofluorometer with excitation at 340 and 380 nm every second. The concentration of intracellular calcium $[Ca^{2+}]_i$ was calculated using $[Ca^{2+}]_i = Kd[R - R_{min}/(R_{max} - R)]$, R being the ratio of the emission intensities measured on excitation at 340 and 380 nM. R_{min} was obtained in the presence of BAPTA-AM (25 mM) 90 min prior to stimulation with sublytic complement and R_{max} upon lysis of the cells with Triton X-100 (0.1%) thereby giving a reading at the different external concentrations of calcium used. The Kd for Fura-2 was taken as 224 nM.

2.7. Establishment of relative intracellular and intravesicular calcium levels using Calcium Green-1-AM

Cells (1×10^6/ml) were treated with 5 μM CG-1-AM for 45 min. Collected MVs (at 1×10^5/ml) and cells were assessed for calcium as previously described [9].

2.8. SDS-PAGE and Western blotting

For cell lysates, RIPA buffer was used (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) in the presence of a protease inhibitor cocktail. Protein content was determined using a bicinchoninic acid protein assay kit (BCA) (Pierce, Thermo Scientific). After removal of insoluble material, a non-reducing sample buffer was added to solubilise the MVs. Electrophoresis of proteins and immunoblotting was carried out as described previously [10].

2.9. Statistical analysis

All readings were carried out in triplicate and experiments repeated three times. The data are represented as mean ± standard error of the mean. Statistical analysis (unpaired t test or 1-/2-way ANOVA) was performed using version 5.0 of the GraphPad Prism software, (GraphPad Software, San Diego, CA). The following significance levels were used: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Cells stimulated through sublytic C5b-9 release MVs through calcium-mediated activation of protein kinase C

Sublytic complement stimulated a greater release of MVs from metastatic PCa, PC3, cells than from an equivalent number of benign prostate PNT2 cells (Fig. 1A) but the MV release increased marginally as the cell number was increased up to 10-fold. We also found that extracellular calcium in the range 0.5–4.0 mM was required for sublytic C5b-9-mediated MV release with a peak occurring at 1.0 mM. This approximates to the typical levels found in extracellular fluid of about 1.2 mM [11,12]; the low level release of MVs at 0 mM Ca^{2+} is presumably constitutive as complement will not activate in the absence of Ca^{2+}.

That MVs were being isolated and analysed is shown in Fig. 1C where NTA reveals a population of MVs in the range 100–550 nm with a modal peak of 260 nm. That the cells were viable following stimulus with C5b-9 is shown in Fig. 1D where it is shown that there are approximately 8% of cells in early apoptosis and only 3% in late apoptosis. MVs were also confirmed by electron microscopy (Fig. 1E), by showing very low level of expression of the exosome marker CD63 and lack of expression of Alix (Fig. 1F) and by showing a typical scatter plot (Fig. 1G) and high degree of PtSer exposition (Fig. 1H).

As shown in Fig. 1I (confirming previous observations [7,12,13]), heat inactivation and C9 depletion of NHS abrogate MV release. This indicates that the deposition of sublytic membrane attack complex (MAC) and influx of calcium (Fig. 3) (and because MV release was also reduced in the presence of EGTA, Fig. 1G), is

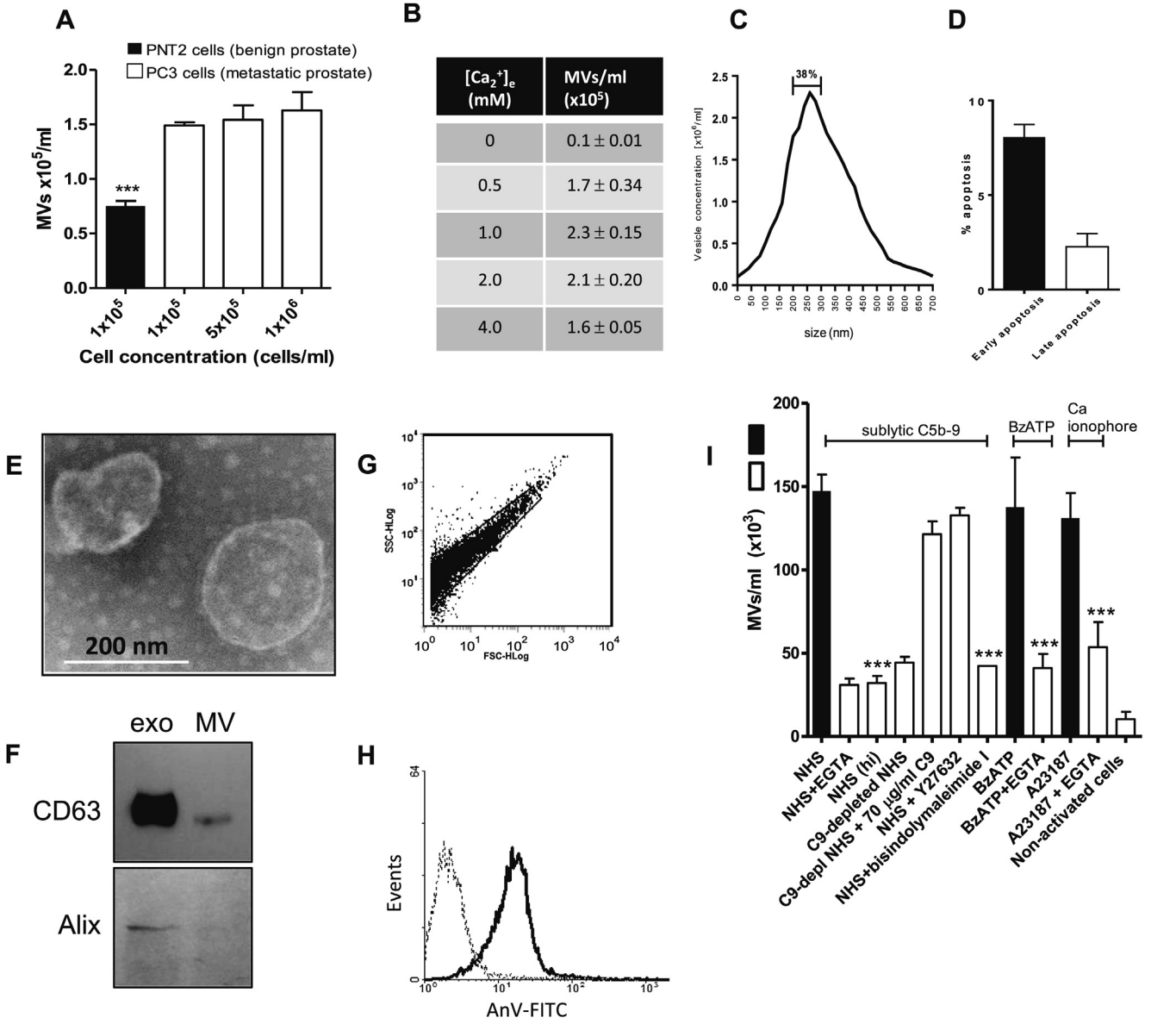

Fig. 1. Microvesiculation from complement-stimulated PC3 cells occurs through activation of protein kinase C. (A) PCa cells release twice as many MVs as benign prostate cells reaching saturated levels with sublytic C5b-9 at 1×10^5 cells/ml. (B) The optimal concentration of extracellular calcium for MV release was 1 mM. (C) MVs were detected by NTA analysis showing a modal peak of 260 nm and 38% of MVs occurring in the range 200–300 nm. (D) 8% of cells were deemed early apoptotic (PS^+/7-AAD$^-$) and 2.3% late apoptotic after complement stimulus. (E) Electron microscopy shows typical MVs and (F) Western blotting confirms low level of CD63 expression and lack of expression of Alix (exosomal markers) on MVs. FSC/SSc scatter plot of MVs (G) and annexin V-labelling (H). In (I) PC3 MVs released from 1×10^6 cells using a variety of stimuli were collected and quantified by NTA. The stimuli were 5% EV-free NHS (5% for 30 min), BzATP (0.1 µM for 20 min), calcium ionophore (A23187), 5 µM for 20 min, and controls (EGTA chelation of Ca^{2+}; heat inactivation; C9-depletion; bisindolymaleimide I and Y27632). MVs were also collected from non-activated PC3 cells grown in complete growth medium (CGM) (which included 5% vesicle-free FBS).

essential for MV formation. MV release was inhibited by the PKC inhibitor bisindoylmaleimide I but not the Rho kinase inhibitor, Y27632. PC3 cells could also be stimulated to microvesiculate, in the presence of calcium, with BzATP, previously reported to stimulate the P2X7 receptor [14], as well as calcium ionophore, A23187 and to generate scatter plots with the same distribution as for MVs obtained in Fig. 1G (cells stimulated with sublytic complement).

3.2. After stimulating cells with sublytic C5b-9 there is a 20 min refractory period during which cells cannot be re-stimulated to release MVs

PC3, LNCap and Du-145 cells (1×10^6) (Fig. 2A–C, respectively) were stimulated with 5% EV-free NHS and 2 mM Ca^{2+} for 15 min. The number of MVs released at this initial stimulation was ~1.8×10^5 MVs/ml. The three PCa cell lines were then rested at 37 °C and re-stimulated with 5% NHS at progressive time points (up to 2 h post initial stimulus) to ascertain how long a recovery period stimulated cells needed to be able to release maximum levels of MVs upon restimulation. We found that the initial stimulus was followed by an approximate 20 min refractory period during which no additional MVs could be released upon restimulation. We conjecture that this is because over the first 20 min after stimulation of cells with sublytic complement there is a continued, albeit decreasing rate of MVs released/ml/min (Fig. 2D). By 20 min, there is no MV release over background meaning that cells are no longer able to vesiculate away any added sublytic MAC. The small increase in MV release at 10 min, probably represents the latent release of

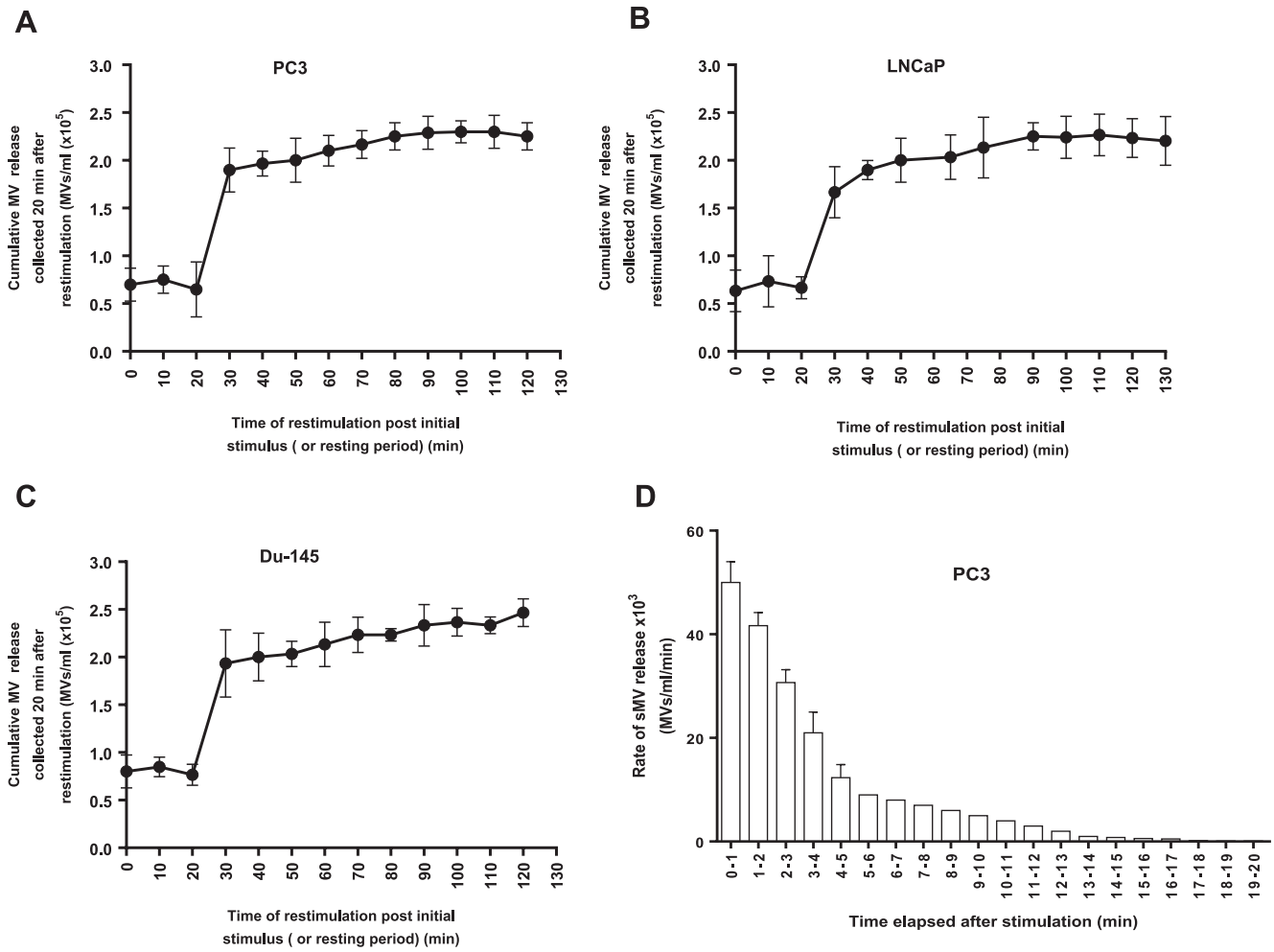

Fig. 2. Cells have a 20 min refractory period before they can be restimulated with sublytic MAC to release MVs. (A–C) PC3, LNCap and Du-145 cells respectively were analysed for subsequent MV production following initial MV release. Cells were stimulated with 5% vesicle-free NHS and MVs collected and quantified (1.8×10^5 MV/ml). The cells were then stimulated with 5% vesicle-free NHS at progressive time points and the MVs collected and quantified. (D) For each of the individual minutes (up to 20 min) following cell stimulation, the MVs released by the end of each of those minutes was quantified to indicate the fall in released MVs/ml/min over time.

MVs from the initial stimulation that is not evident at 20 min. After 30 min resting, MVs were released upon restimulation at levels approximating the initial stimulatory event. This level continued to rise very slightly when cells were restimulated at times up to 120 min after the original stimulus.

3.3. MVs are released from cells, as raised cytosolic concentrations of calcium in cells fall to resting levels

Intracellular calcium is stored within organelles until required, or may enter from outside the cell through various channels. As an excess of calcium may cause a multitude of undesirable effects, a cell may need to expel calcium effectively. We already know that microvesiculation is involved in the removal of sublytic MAC from cells [15]. If MVs are found to harbour Ca^{2+}, the restoration of internal calcium levels by microvesiculation might thus play a role in calcium homeostasis. Free intracellular calcium by binding to cytoskeletal associated enzymes such as calpain, leads to cytoskeletal cleavage and the depolymerisation of F-actin to the poorly organised G-actin and the eventual formation and release of MVs.

To see whether the sublytic complement deposition on PC3 cells causes an increase in $[Ca^{2+}]_i$, which stimulates MV release, the cells were preloaded with fura 2-AM and this was measured spectrophotometrically. After a rise of 79 nMs^{-1} over 11 s and reaching a peak $[Ca^{2+}]_i$ of 920 nM, the concentration of cytosolic calcium fell steadily at 2.4 nMs^{-1} over the following 109 s (Fig. 3A), baseline levels (50–100 nM) being reached within 10–15 min (Fig. 3B). Concomitantly, MV release continued to rise, reaching a plateau of just under 1.7×10^5 MVs/ml (7× that released from resting cells) in about 8 min.

Whether MV release from, or fusion with cells could impact at all on cellular calcium levels, could only be ascertained with an estimate of microvesicular calcium concentration. We anyway expected MVs to be calcium carriers as they harbour various non-ER lumen calcium-binding proteins such as those of the S100 family, troponin C, calmodulin and calbindins [16], also believed to be involved in calcium homeostasis.

3.4. MVs released from stimulated cells carry intravesicular calcium

To gain an insight as to whether MVs carry Ca^{2+} (initially in a non-quantitative manner) calcium Green^{TM-1}-AM (CG-AM) dye which passes freely across the plasma membrane as a steroid-based molecule, and is big enough (>30,000 Mr) and comparatively positively charged not to leak through MAC pores, was used. The dye component is cleaved from the steroid by intracellular

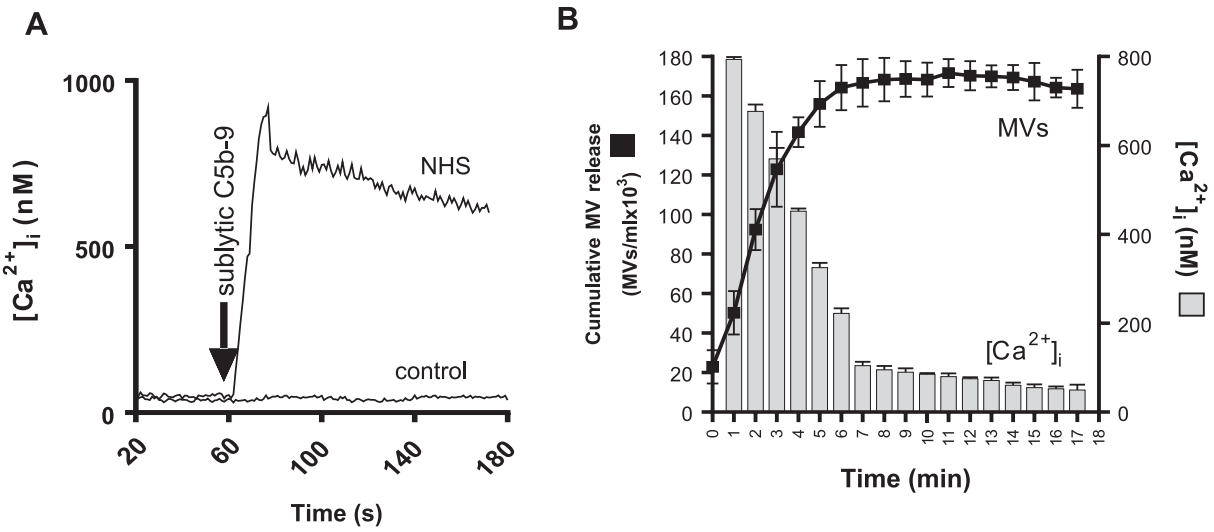

Fig. 3. The rise in intracellular calcium in complement-stimulated cells recovers to basal levels within 10–15 min by which MV release has ceased. (A) Spectrofluorometer measurements of intracellular calcium concentrations were made using fura 2-AM upon stimulation of cells with 5% NHS. Control cells were pretreated for 30 min with 10 μM BAPTA-AM. (B) Over a 17 min period after stimulation of cells, the concentration of intracellular calcium was monitored using a spectrofluorometer every minute using fura 2-AM, as above, as well as the concentration of MVs released.

esterases, causing the dye to accumulate. Fluorescence was measured with a FLUOstar omega microplate reader. Cells loaded with CG-AM were then stimulated for MV release. MVs appear to contain different levels of Ca^{2+} when directly compared at a 1:1 ratio (Fig. 4A) depending on how they are released. That MVs released from stimulated cells contain more Ca^{2+} than MVs released constitutively from cells is to be expected as they derive from cells with higher $[Ca^{2+}]_i$. It also implies that MVs are not 'leaky' to Ca^{2+} even though as we have observed, they do carry C5b-9 [17]. For now we cannot comment on sources of $Ca^{2+}{}_i$ beyond MAC pores, such as mitochondria or ER. Calcium channels can be ruled out as verapamil did not significantly reduce $[Ca^{2+}]_i$ levels (Fig. 4B). Notably, this result also shows the presence of the unspecific, cytosolic active esterase (able to cleave the Calcium Green from its steroid carrier) within the MV, to allow for dye accumulation.

To estimate $[Ca^{2+}]_i$ in MVs quantitatively we used Fura-2 AM. Five minutes after stimulating fura-2 AM-loaded cells with sublytic c5b-9, by which time MV release had reduced to 1/5th of its initial release rate (Fig. 2D) and cellular $[Ca^{2+}]_i$ was ~350 nM (Fig. 3B), we found $[Ca^{2+}]_i$ in PC3 MVs to be 430 nM which compared to 480 nM within cells. The true intravesicular calcium concentration may of course vary considerably depending how soon after stimulus the MV is released. We confirmed that calcium influx was through sublytic C5b-9 pores and not calcium channels by seeing little drop in $[Ca^{2+}]_i$ in PC3 cells pretreated with verapamil and a maintenance of basal levels in calcium-free medium (Fig. 4B). Calcium ionophore (A23187) treatment also increased intracellular calcium levels which could be abrogated with BAPTA-AM.

Such concentrations within released MVs may theoretically influence the restoration of normal $[Ca^{2+}]_i$, but whether such levels could impact on cellular concentrations of calcium in practise would depend on the numbers of MVs released from cells and this could range from 3800 to over 25,000 MVs per cell/48 h. Assuming 25,000 MVs released over 48 h and the $[Ca^{2+}]_i$ to be 430 nM per MV and up to 920 nM per stimulated cell, it seems over the few minutes that it takes for $[Ca^{2+}]_i$ to be restored to resting levels and at the MV release rates we measured, upon stimulation of cells with sublytic complement, MVs could at most play only a minor role in calcium homeostasis. However, the physical removal of MAC pores [15,18] as we showed elsewhere [17] could itself curtail increases in $[Ca^{2+}]_i$ in the short term. Indeed in some of the original work on MVs, suggesting their protective roles against complement-mediated lysis, it was suggested that the permeability changes in cells due to MAC pores, were temporary, existing only until MVs had been released [19]. Similarly, the addition of MVs to cells may contribute to modest increases in cytosolic $[Ca^{2+}]$ but may also involve the transfer of MAC or calcium channels to recipient cells, a process which has not hitherto been reported in the literature.

4. Discussion

Microvesiculation may be triggered from cells upon deposition with sublytic complement (Membrane Attack Complex, C5b-9) by increasing $[Ca^{2+}]_i$, as it rises to ≥300 nM. We found that a repeated stimulus, in this case with sublytic C5b-9 of PCa cells, PC3, LNCaP and Du-145, is unable to allow microvesiculation to re-occur until 20 min have elapsed. After this, the cells are able to take a repeat stimulus and to release MVs once more. Refractory periods where cells lose their sensitivity to calcium stimulation are common, as with mast cells permeabilized with streptolysin-O that lose their capacity to be stimulated over 20–30 min [20]; Listeriolysin O, the toxin from *Listeria monocytogenes* has the same effect on mast cells [21]. The latter work further showed the refractory stimulation by calcium to be due to reduced intracellular release of calcium rather than to be influenced by extracellular calcium influx. Although we found in calcium-free conditions MAC to not stimulate raised intracellular calcium, we cannot rule out the role of ER-derived calcium released through IP_3R channels after MAC stimulation, which could in turn trigger apoptosis through mitochondrial damage [22].

Despite increasing levels of MV release over 17 min occurring alongside decreasing $[Ca^{2+}]_i$, it is still not possible to say whether, in addition to the ongoing intracellular sequestration of Ca^{2+}, microvesiculation *per se* contributes significantly to the homeostasis of cytosolic $[Ca^{2+}]$. Even though intravesicular concentrations of calcium which we estimated at 430 nM are higher than basal levels in unstimulated cells (50–100 nM) and about half that of newly stimulated cells (920 nM), they may be too low to make a difference (30 μM Ca^{2+} being required for half-maximal activation

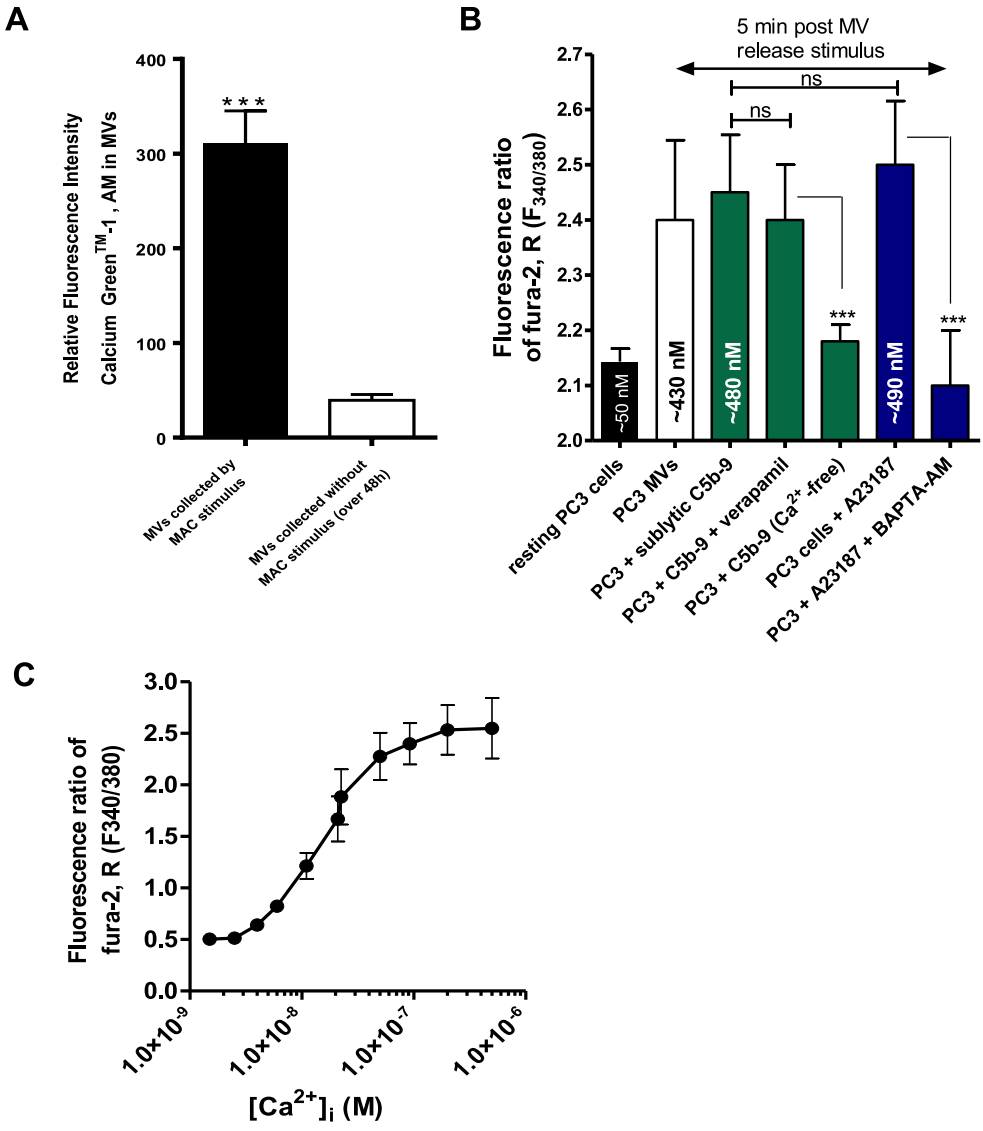

Fig. 4. MVs released from complement-stimulated PCa cells carry calcium. (A) Calcium Green-1-AM (Invitrogen) was incubated with PC3 to bind to intracellular calcium. When exciting with 5% EV-free NHS, resultant membrane pores led to MV release. MVs from Calcium Green-1-AM-loaded cells were collected and the relative Calcium Green fluorescence intensity for the intravesicular CG-1-AM measured with the FLUOstar Ω multiplate reader, stimulating at A_{485} and reading at A_{520}. MVs released following stimulation (of 1×10^5/ml cells) show significantly higher levels of calcium fluorescence than for MVs released without stimulation (from 1×10^5/ml, collected over 48 h). (B) The fluorescence ratio of fura-2 in PC3 cells (1×10^4 cells), 5 min after stimulus with C5b-9 increased, only in the presence of extracellular calcium, equating to a rise from 50 to 480 nM; this could not be reduced by blocking calcium channels. The estimated $[Ca^{2+}]_i$ in MVs was estimated at 430 nM. (C) Concentrations of calcium were calculated as described in Materials and Methods and also by interpolation on a calibration curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of calpain-1 [23]) unless in local micro-environments, adjacent to where MAC pores are formed, levels are much higher ([15,23,24]). Localised vesiculation may then be more effective in restoring $[Ca^{2+}]_i$ concentrations. Another consideration is that MV release itself might eventually control levels of MAC pores by removing them on MVs. Furthermore, although levels of MV release (at maximum 25,000 per cell/48 h) are unlikely *per se* to make an impact on raised $[Ca^{2+}]_i$ over 30 min, the contribution of exosomes was not considered.

Breaches of the plasma membrane for example with streptolysin O, a bacterial pore forming toxin or sublytic C5b-9 pores may lead to apoptosis [25]; we recently also described Extremely Low Frequency Magnetic Fields to induce surface pores that stimulate MV release through raised $[Ca^{2+}]_i$ [9]. We believe that just as blebbing of the plasma membrane averts a fatal proteolysis and activation of phospholipases in the cell, by blocking off raised $[Ca^{2+}]_i$ [26], that MV release may form an alternative, but somewhat similar mechanism of protecting against a necrotic death by releasing or confining pore-forming proteins and of dealing with elevated levels of calcium.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.061.

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