Inhibition of germinal vesicle breakdown by antioxidants and the roles of signaling pathways related to nitric oxide and cGMP during meiotic resumption in oocytes of a marine worm

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Abstract

In mammalian oocytes, cAMP elevations prevent the resumption of meiotic maturation and thereby block nuclear disassembly (germinal vesicle breakdown (GVBD)), whereas nitric oxide (NO) and its downstream mediator cGMP can either inhibit or induce GVBD. Alternatively, some invertebrate oocytes use cAMP to stimulate, rather than inhibit, GVBD, and in such cases, the effects of NO/cGMP signaling on GVBD remain unknown. Moreover, potential interactions between NO/cGMP and AMP-activated kinase (AMPK) have not been assessed during GVBD. Thus, this study analyzed intraoocytic signaling pathways related to NO/cGMP in a marine nemertean worm that uses cAMP to induce GVBD. For such tests, follicle-free nemertean oocytes were stimulated to mature by seawater (SW) and cAMP elevators. Based on immunoblots and NO assays of maturing oocytes, SW triggered AMPK deactivation, NO synthase (NOS) phosphorylation, and an NO elevation. Accordingly, SW-induced GVBD was blocked by treatments involving the AMPK agonist AICAR, antioxidants, the NO scavenger carboxy-PTIO, NOS inhibitors, and cGMP antagonists that target the NO-stimulated enzyme, soluble guanylate cyclase (sGC). Conversely, SW solutions combining NO/cGMP antagonists with a cAMP elevator restored GVBD. Similarly, AICAR plus a cAMP-elevating drug reestablished GVBD while deactivating AMPK and phosphorylating NOS. Furthermore, sGC stimulators and 8-Br-cGMP triggered GVBD. Such novel results indicate that NO/cGMP signaling can upregulate SW-induced GVBD and that cAMP-elevating drugs restore GVBD by overriding the inhibition of various NO/cGMP downregulators, including AMPK. Moreover, considering the opposite effects of intraoocytic cAMP in nemerteans vs mammals, these data coincide with previous reports that NO/cGMP signaling blocks GVBD in rats.

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Introduction

Based on several lines of evidence, signaling pathways involving the reactive nitrogen species (RNS) nitric oxide (NO) can play a key role in regulating oocyte maturation (Zhang *et al.* 2007, 2009, Pandey *et al.* 2010, Tsafirri & Dekel 2010). However, analyses of mammalian oocytes have sometimes presented conflicting data regarding the functions of NO and its common downstream mediator, cGMP (LaPolt 2002), particularly during the resumption of meiotic maturation, as the oocyte undergoes nuclear disassembly (germinal vesicle breakdown (GVBD)); Downs 2010.

Thus, mammalian GVBD is often stimulated by antioxidants that can counteract the effects of RNS and reactive oxygen species (ROS; Sun *et al.* 2000, Tao *et al.* 2004a, 2010, Nadri *et al.* 2009), and consistent with these findings, GVBD is prevented or delayed by treatments that elevate intraoocytic NO/cGMP levels (Törnell *et al.* 1990, Faerge *et al.* 2001, Nakamura *et al.* 2002, Tao *et al.* 2005, Zhang *et al.* 2005, Viana *et al.* 2007,

Sela-Abramovich *et al.* 2008). Conversely, in other studies of mammals, antioxidants block GVBD (Takami *et al.* 1999), and pathways involving NO and cGMP promote GVBD (Hubbard & Price 1988, Jablonka-Shariff & Olson 2000, Sengoku *et al.* 2001, Tao *et al.* 2004b, Huo *et al.* 2005, Chmelikova *et al.* 2010, Amale *et al.* 2011). Although species-specific differences might account for some of these discrepancies, both inhibitory and stimulatory effects of NO signaling have been reported for the same oocytes (Bu *et al.* 2003, Bilodeau-Goeseels 2007). Hence, the conflicting results may also involve such confounding factors as the type of maturation-inducing stimulus that is applied, the presence or absence of follicle cells, and/or the ability of high vs low NO levels to inhibit or induce GVBD by targeting differing downstream pathways (Faerge *et al.* 2001, Bu *et al.* 2003, 2004, Thaler & Epel 2003, Bilodeau-Goeseels 2007, Abbasi *et al.* 2009).

Regardless of the reasons for such differences, it should be noted that oocytes of mammals, other vertebrates, and starfish are typically prevented from

maturing by high levels of intraoocytic cAMP (Voronina & Wessel 2003, Sun *et al.* 2009, Jaffe & Norris 2010). Conversely, instead of blocking maturation, intraoocytic cAMP elevations induce GVBD in some marine invertebrates (Deguchi *et al.* 2011), including worms of the phylum Nemertea (Stricker & Smythe 2001). In addition, the energy sensor and cell-cycle regulator AMP-activated kinase (AMPK; Carling 2005) can differentially affect maturation, since intraoocytic AMPK activation elicits GVBD in mice (Downs *et al.* 2002, Chen *et al.* 2006, Chen & Downs 2008) but blocks GVBD in nemerteans (Stricker *et al.* 2010a,b; Stricker 2011).

Currently, the functions of NO/cGMP signaling in oocytes that use cAMP elevations to trigger, rather than inhibit, GVBD are unknown. Similarly, potential interactions that might occur between NO/cGMP and AMPK have not been investigated in oocytes. Thus, to assess the roles played by NO, cGMP, cAMP, and AMPK in a case where intraoocytic cAMP induces GVBD, this study analyzed follicle-free oocytes of the marine nemertean worm *Cerebratulus* that were stimulated by seawater (SW) and cAMP-elevating drugs in the presence or absence of antioxidants, an AMPK stimulator, or other modulators of NO/cGMP signaling.

Results

Blockage of SW-induced GVBD by antioxidants

Since nemertean oocytes typically lack follicle cells (Stricker *et al.* 2001), antioxidant tests involved follicle-free oocytes that were initially kept immature by preincubation in calcium-free SW (CaFSW). Such oocytes were then transferred to SW solutions of antioxidants (ascorbic acid, butylated hydroxyanisole (BHA), gallotannin, *N*-acetylcysteine (NAC), or tempol), which were chosen in order to compare with previous studies that subjected mammalian oocytes and somatic cells to these and related compounds (Sun *et al.* 2000, Ali *et al.* 2003, Tao *et al.* 2004a, Dumollard *et al.* 2007, Kim *et al.* 2009). In such tests, SW-induced GVBD in nemerteans was blocked in a dose-dependent fashion, and >50% inhibition occurred at drug concentrations that were comparable with those used on other oocytes (Sun *et al.* 2000, Ali *et al.* 2003, Tao *et al.* 2004a,b; Fig. 1A). Moreover, such blockages were not due to oocyte morbidity, since coincubations with antioxidants plus a cAMP elevator restored GVBD (Fig. 1B, C and D).

To verify that antioxidant-treated oocytes were prevented from maturing by mechanisms resembling those previously reported for *Cerebratulus* (Stricker & Smythe 2003, 2006a, Stricker *et al.* 2010a,b), AMPK and maturation-promoting factor (MPF) were assessed via phospho-specific antibodies. Based on such tests, all of the assayed antioxidants (BHA, gallotannin, NAC, and tempol) maintained high activity of the GVBD inhibitor AMPK while keeping MPF, a key inducer of GVBD

Figure 1 (A) Antioxidants (aa, ascorbic acid; BHA, butylated hydroxyanisole; gallo, gallotannin; NAC, *N*-acetylcysteine; tem, tempol) block seawater (SW)-induced GVBD in a dose-dependent manner. (B and C) Conversely, such blockage is reversed after coadding 5 μM of the cAMP elevator forskolin (fors) to 200 μM aa, 500 μM BHA, 100 μM gallo, 150 μM NAC, or 150 μM tem. (D) Accordingly, blots probed with antibodies to active AMP-activated kinase (AMPK) and active maturation-promoting factor (MPF) show forskolin cotreatment restores both AMPK deactivation and MPF activation. bf, before stimulation; CaFSW, calcium-free seawater; GV, germinal vesicle. Asterisks mark values significantly ($P < 0.05$) lower than in SW controls.

(Kishimoto 2003), from being activated. Conversely, coaddition of a cAMP elevator restored GVBD by allowing both AMPK deactivation and MPF activation (Figs 1D and 2D).

Role of NO during GVBD

Since each of the tested antioxidants can lower NO levels in somatic cells (Bergamini *et al.* 2001, Linares *et al.* 2008, Kim *et al.* 2009, Harput *et al.* 2011), the

Figure 2 (A and B) The NO scavenger cPTIO blocks SW-induced GVBD, and inhibition induced by 200 μM cPTIO is reversed by cotreatment with the cAMP elevator forskolin (for or fors, at 5 μM). (C and D) 200 μM cPTIO (cp) blocks AMPK deactivation and MPF activation as effectively as antioxidants (ao; pooled data for 500 μM BHA, 100 μM gallotannin, 150 μM NAC, and 150 μM tempol), and such blockage is rescued by coadding cAMP elevators ('+cAMP', pooled data for 5 μM fors, 200 μM IBMX and 1 μM 5HT). (E) Nitric oxide synthase (NOS) inhibitors L-NAME (50 mM) and DPI (30 μM) downregulate SW-induced GVBD, particularly following a 2-h preincubation (preinc) in calcium-free seawater (CaFSW), and such inhibition is reversed by the cAMP elevator forskolin (fors). (F and G) Based on immunoblots using a phospho-specific antibody to active endothelial NOS (eNOS), the increased NOS signal that normally occurs during SW-induced GVBD is blocked by the AMP-activated kinase (AMPK) activator AICAR (333 μM) and then restored, along with GVBD, by cotreatment with AICAR plus a cAMP elevator ('+cAMP', pooled data for 5 μM forskolin, 200 μM IBMX and 1 μM 5HT). Accordingly, such oocytes exhibit a decrease in inactive maturation-promoting factor (MPF). bf, before stimulation. Asterisks mark values significantly ($P < 0.05$) lower than in SW controls. Xs mark values significantly ($P < 0.05$) lower than in immature oocytes before treatment (BF).

effect of NO signaling on GVBD was assessed by means of the relatively specific NO scavenger carboxy-PTIO (cPTIO; *Akaike et al. 1993*). In such tests, transfer of immature oocytes from CaFSW directly into SW solutions of cPTIO significantly ($P < 0.05$) decreased GVBD (Fig. 2A), and this inhibition was rescued by coaddition of a cAMP elevator (Fig. 2B). Moreover, as with antioxidants, cPTIO significantly ($P < 0.05$) reduced AMPK deactivation while preventing MPF activation, whereas coadding cAMP elevators reversed such effects (Fig. 2C and D).

Similarly, in oocytes treated with either 50 mM L-NAME, a broad-spectrum inhibitor of NO synthases (NOSs), or 30 μM of diphenyleneiodonium (DPI), a blocker of endothelial or inducible forms of NOS (eNOS), GVBD was reduced in response to stimulation by SW, but not by SW plus a cAMP elevator (Fig. 2E). However, perhaps because of lower oolemmal permeabilities compared with cPTIO, oocytes had to be preincubated for 1–2 h in CaFSW plus the NOS inhibitor

to yield a significant ($P < 0.05$) reduction in GVBD by 2 h postincubation in SW solutions of the drugs (Fig. 2E). Moreover, although maturation continued to be blocked in DPI-treated oocytes examined the next morning, for undetermined reasons, GVBD was only delayed in L-NAME-treated specimens, as a majority eventually underwent GVBD following overnight incubation (data not shown).

Nevertheless, consistent with such downregulation of GVBD, immature oocytes that had been frozen before SW stimulation or after incubation in SW plus the AMPK stimulator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) exhibited a relatively weak band at ~130 kDa, when probed with a phospho-specific antibody against active mammalian eNOS (Fig. 2F and G). Conversely, the putative 130-kDa NOS signal increased significantly ($P < 0.05$) in mature oocytes that were stimulated with SW or SW solutions of AICAR plus a cAMP elevator (Fig. 2F and G). Moreover, as quantified previously (*Stricker et al. 2010a*),

Figure 3 (A and B) Calcium-free seawater (CaFSW) solutions of NO donors sodium nitroprusside (SNP) and DPTA NONOate fail to trigger GVBD, and at higher doses such treatments reduce GVBD compared with controls in CaFSW alone. Asterisks mark values significantly ($P < 0.05$) lower than in CaFSW controls.

cotreatments with AICAR plus cAMP elevators also promoted AMPK deactivation (data not shown).

Accordingly, by GVBD onset at 30 min poststimulation, SW-treated oocytes elevated their NO signals $26 \pm 18\%$ ($n=5$) over pretreatment baselines, based on total nitrate/nitrite detected by the Griess reagent. In comparison, immature cPTIO-treated oocytes exhibited substantially lower NO values in two of three cases, although the $12 \pm 33\%$ increase over baseline ($n=3$) for all three batches of cPTIO-treated oocytes turned out not to be significantly less than in SW controls.

To determine if NO donors can trigger GVBD, oocytes were incubated in CaFSW solutions of the NO donor sodium nitroprusside (SNP) or dipropyleneetriamine NONOate (DPTA NONOate). However, the slightly elevated GVBD levels triggered by low NO donor doses were not significantly greater than in CaFSW alone, and higher drug doses actually yielded progressively less maturation (Fig. 3A and B). Moreover, significantly lower ($P < 0.05$) GVBD levels such as generated by 10 mM SNP or 2 mM DPTA NONOate were reversed by coaddition of cAMP elevators (data not shown), thereby ruling out morbidity effects.

Role of cGMP during GVBD

Given that a major cellular target stimulated by NO is soluble guanylate cyclase (sGC) that in turn can generate cGMP (Denninger & Marletta 1999), the role of cGMP during GVBD was tested by pretreating oocytes with CaFSW solutions of three sGC inhibitors: 100 μM 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), 400 μM methylene blue (MB), and 50 μM

4-H-bromo-1,2,4-oxadiazole(3,4-aldea)benz(b)oxazin-1-one (NS2028; Hwang *et al.* 1998, Olesen *et al.* 1998). In such cases, ODQ and MB preincubations significantly reduced ($P < 0.05$) GVBD in SW solutions of the inhibitors at 2 h posttreatment (Fig. 4A) or when checked the next morning (data not shown). Moreover, such reductions were reversed by coaddition of a cAMP elevator (Fig. 4A). A similar pattern was also observed for NS2028, although GVBD following NS2028 preincubation was only marginally lower ($P < 0.10$) than in SW controls.

Consistent with these findings, immature oocytes that were treated for 2 h with CaFSW solutions of 250 μM 3-bromo-3,4,4-trimethyl-3,4-dihydrodiazete 1,2-dioxide (DD1), 75 μM 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol (YC1), or 150 μM protoporphyrin IX to activate sGC (Evgenov *et al.* 2006) exhibited elevated GVBD levels ($P < 0.05$) compared with controls in CaFSW alone (Fig. 4B). Similarly, moderately higher GVBD relative to CaFSW controls ($P < 0.10$) occurred for six pairings of CaFSW vs CaFSW plus 5 μM vinpocetine or 20 μM zaprinast (Fig. 4C), two phosphodiesterase (PDE) inhibitors that can elevate cGMP levels by downregulating cGMP degradation (Ahn *et al.* 1989, Choi *et al.* 2002).

Figure 4 (A) The soluble guanylate cyclase (sGC) inhibitors ODQ (100 μM), methylene blue (meth blue, 400 μM) and NS2028 (50 μM) downregulate seawater (SW)-induced GVBD, particularly following a 2-h preincubation (preinc) in calcium-free seawater (CaFSW), whereas such inhibition is reversed by cotreatment with 5 μM of the cAMP elevator forskolin (for). (B and C) Conversely, CaFSW solutions of sGC stimulators (250 μM DD1, 75 μM YC1, or 150 μM protoporphyrin IX (PPIX)) significantly ($P < 0.05$) increase GVBD over levels in CaFSW alone, whereas cGMP-targeted PDE inhibitors (20 μM zaprinast (zap), 5 μM vinpocetine (vinp)) increase GVBD only moderately ($P < 0.10$), compared with CaFSW controls. Asterisks mark values significantly ($P < 0.05$) lower than in SW controls. Plus signs mark values significantly ($P < 0.05$) higher than in CaFSW controls.

Figure 5 (A) 2.5 mM 8-Br-cGMP dissolved in pH 8 calcium-free seawater (CaFSW) fails to stimulate GVBD at 2 h posttreatment but promotes more GVBD after overnight incubation, suggesting that drug permeability may be suboptimal. (B–D) To facilitate delivery of 8-Br-cGMP, pH 7.4 and pH 8.6 CaFSWs were tested. However, because pH 7.4 CaFSW elevated GVBD in the absence of 8-Br-cGMP (B), only pH 8.6 CaFSW was used for dissolving 8-Br-cGMP. Such pH 8.6 solutions of 2.5 mM 8-Br-cGMP triggered SW-like levels of GVBD either (C) after overnight or (D) 6 h incubations, while intermediate, or essentially no, GVBD occurred with 0.5 mM or 0 mM 8-Br-cGMP respectively. + mark values significantly ($P < 0.05$) lower than in SW controls. Plus signs mark values significantly ($P < 0.05$) higher than in CaFSW controls.

As a more direct test of cGMP's ability to stimulate GVBD, immature oocytes were treated with the relatively membrane-permeable and PDE-resistant 8-bromo form of cGMP (8-Br-cGMP), which was initially assessed at a 2.5 mM dose in pH 8 CaFSW. At 2 h postincubation, such treatments did not increase GVBD, but when checked the next morning, 8-Br-cGMP-treated specimens had undergone significantly ($P < 0.05$) more GVBD than did CaFSW controls (Fig. 5A), suggesting that the delayed reaction may have resulted from a retarded entry of 8-Br-cGMP.

Accordingly, given that acidified or alkalinized SW can enhance cyclic nucleotide delivery into oocytes (Yamashita 1988, Deguchi *et al.* 2011), pH 7.4 and 8.6 CaFSW solutions were tested and found in the absence of added 8-Br-cGMP to increase and decrease, respectively, GVBD, compared with pH 8 CaFSW (Fig. 5B). Thus, to avoid the elevated basal levels of GVBD triggered by acidified CaFSW, 2.5 mM 8-Br-cGMP was dissolved in pH 8.6 CaFSW that was alkalinized with NaOH and filtered before use. At 2 h posttreatment, such solutions caused noticeably more GVBD than in pH 8.6 CaFSW controls. Moreover, when examined the next morning, 18/19 of the oocyte batches treated with 8-Br-cGMP in pH 8.6 CaFSW exhibited at least 95% GVBD, thereby matching averages in response to SW and exceeding ($P < 0.05$) control values in CaFSW alone (Fig. 5C). Similarly, in time-lapse monitoring of six batches, nearly all oocytes treated with 2.5 mM 8-Br-cGMP underwent GVBD by 6 h, whereas 0.5 mM 8-Br-cGMP and pH 8.6 CaFSW caused intermediate, and essentially no, increases in GVBD, respectively, over that time period (Fig. 5D). In addition, such GVBD was not due to a pH change induced by 8-Br-cGMP, since the initial pH after dissolving 2.5 mM 8-Br-cGMP in pH 8.6 CaFSW was 8.5–8.6, and the next morning, the pH of both the 8-Br-cGMP solution and CaFSW control measured ~ 8.4 .

Discussion

As noted previously for the antioxidant resveratrol (Stricker *et al.* 2010a), this study reveals that various other antioxidants also inhibit SW-induced GVBD in nemertean oocytes, whereas coaddition of a cAMP elevator restores maturation. Currently, there is no direct evidence that antioxidant treatments decrease NO in nemertean oocytes by either downregulating NOS activity or scavenging NO, as demonstrated for somatic cells (Bergamini *et al.* 2001, Linares *et al.* 2008, Kim *et al.* 2009, Harput *et al.* 2011). Moreover, even if the antioxidants had been shown to reduce intraoocytic NO, such findings would not preclude the possibility that these broadly acting drugs block GVBD via effects on other targets, including ROS (Nakagawa *et al.* 2010). Nevertheless, the antioxidant-mediated inhibition of GVBD is at least consistent with NO promoting GVBD in SW-stimulated oocytes.

Accordingly, SW-induced GVBD is downregulated by the NO scavenger cPTIO or the NOS inhibitors L-NAME and DPI, whereas such effects are reversed by coadding a cAMP elevator. Moreover, for immature oocytes frozen before SW stimulation or after treatment with SW solutions of the AMPK activator AICAR, blots probed with an antibody to active eNOS indicate that putative NOS activity is low compared with postmaturation levels in SW or SW solutions of AICAR plus cAMP elevators. It should be noted, however, that although nemertean oocytes yield a band near the appropriate MW for eNOS and are prevented from maturing by an e/iNOS inhibitor, NOS enzymes of invertebrates (Palumbo 2005) differ from vertebrate forms and may not be readily categorized by the three-isotype scheme applied to vertebrates (Gonzalez-Domenech & Munoz-Chapuli 2010). Nevertheless, the NOS inhibitors used in this study have also been employed in other tests of invertebrate NO signaling pathways (e.g. Moroz *et al.* 1996, Bishop *et al.* 2008), and several lines of evidence

presented here collectively suggest that SW triggers AMPK deactivation and phosphorylation of some type of NOS. Conversely, the AMPK agonist AICAR can apparently inactivate NOS, as noted for somatic cells (Pilon *et al.* 2004), whereas cAMP elevators override AICAR's effects to restore AMPK deactivation, NOS phosphorylation, and GVBD.

In addition, NO assays reveal a pre-GVBD increase in NO. Exactly why cPTIO does not yield significantly lower NO signals than in SW controls remains unknown. However, in two of three cases, such a relationship is observed, whereas the third case with a much higher NO signal for the cPTIO treatment could represent true variability or an artifactual reading, possibly due to incomplete removal of interfering absorption from the darkly colored cPTIO. Nevertheless, consistent with SW-induced NO increases and GVBD blockages by NOS antagonists and cPTIO, inhibitors of the NO target sGC routinely prevent GVBD, collectively suggesting that SW-induced GVBD relies on an NO-mediated cGMP increase.

Conversely, two NO donors fail to elevate GVBD, perhaps indicating that NO signaling is necessary for SW-induced maturation but insufficient by itself to trigger GVBD. However, preliminary tests using the peroxynitrite and NO generator Sin-1 (3-morpholino-sydnonimine) at 2.5 mM yield ~50% GVBD, which more than doubles the value of CaFSW controls (data not shown). Thus, it is possible that the precise types or doses of NO donors for effectively inducing nemertean GVBD have yet to be fully assessed. Moreover, to prevent spontaneous GVBD in SW, NO donors had to be dissolved in CaFSW. Accordingly, in pig oocytes, parthenogenesis induced by NO donors is abolished by the calcium channel blocker verapamil, suggesting that calcium influx is required for optimal NO signaling (Petr *et al.* 2005). Thus, CaFSW solutions of NO donors may not fully mimic NO signaling in calcium-containing SW, and/or they might reduce GVBD due to overly elevated NO (Bu *et al.* 2003), two possibilities that could be tested by monitoring GVBD and NO for various NO donor doses in CaFSW vs SW, with or without added NOS inhibitors.

In any case, CaFSW solutions of sGC stimulators consistently induce GVBD, which in turn shows that activating a downstream target of NO can trigger GVBD in the absence of SW.

Accordingly, 8-Br-cGMP fully stimulates GVBD in CaFSW. The fact that compared with SW, 8-Br-cGMP requires increased pH and a longer lag time until GVBD onset could be due to suboptimal oolemmal permeability of 8-Br-cGMP or the ability of SW to elicit GVBD in a timely fashion via other maturation-inducing signals than just cGMP. Additional experiments such as those involving 8-Br-cGMP microinjections are needed to distinguish between these possibilities. Similarly, further analyses are required to determine if cGMP can also be

elevated by particulate GC signaling (Sandberg *et al.* 1993, Zhang *et al.* 2005) and if NO's effects on nemertean oocytes fail to involve protein kinase G (PKG) activation and/or can utilize cGMP-independent mechanisms (Bilodeau-Goeseels 2007, Wang *et al.* 2008).

As for potential cGMP–cAMP interactions during nemertean GVBD, data presented here suggest two scenarios that are outlined in Fig. 6. One possibility is that an SW-induced cGMP elevation may inhibit PDE to increase cAMP as demonstrated for mice (Norris *et al.* 2009, Zhang *et al.* 2010). This cAMP rise could in turn deactivate AMPK, perhaps via PKA-mediated S485/491 phosphorylation on AMPK (Stricker *et al.* 2010a), although AMPK deactivation due to a drop in AMP as cAMP levels rise cannot be precluded. In either case, however, a PDE downregulation would be opposite to the cGMP-mediated stimulation of PDE activity in *Xenopus* oocytes (Sandberg *et al.* 1993), where cGMP elevations can lower cAMP levels and enhance progesterone-induced GVBD, perhaps by targeting a PDE2 isotype (Sandberg *et al.* 1993), rather than a PDE3 form, such as found in mice (Vaccari *et al.* 2009).

However, if SW-stimulated cGMP elevations simply increase cAMP to elicit nemertean GVBD, it is unclear why inhibitors of the cAMP target PKA do not equally affect GVBD during treatments with SW vs cAMP elevators (Stricker & Smythe 2001, 2006a). Furthermore, along with examples noted here, various modulators prevent GVBD in SW, but not in the presence of cAMP elevators (Stricker & Smythe 2006a,b; Stricker 2009a,b; Stricker *et al.* 2010a,b), which in turn would suggest that the targets of these modulators all occur upstream to the cAMP rise, if in fact SW-stimulated GVBD proceeded exclusively via a cGMP-mediated cAMP elevation. However, Cdc25 inhibitors also block GVBD induced by SW, but not by cAMP elevators (Stricker *et al.* 2006a),

Figure 6 Possible interactions of intraoocytic signaling pathways related to NO/cGMP during nemertean GVBD, showing putative targets of pharmacological modulators that are able to inhibit maturation when oocytes are stimulated by seawater (SW), but not when stimulation involves a cAMP elevator. Note: nemertean oocytes undergo non-follicular oogenesis, and tests outlined here utilize dejellied, isolated oocytes. Thus, NO/cGMP elevations described in this paper are not mediated by somatic cells, as in mammals, but are derived from signaling pathways in the oocytes themselves. aa, ascorbic acid; AMPK, AMP-activated kinase; BHA, butylated hydroxyanisole; gallo, gallo-tannin; MPF, maturation-promoting factor; NAC, N-acetylcysteine; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; tem, tempol.

even though Cdc25 is commonly regulated by PKA downstream to cAMP (Duckworth *et al.* 2002, Zhang *et al.* 2008).

Thus, as an alternative mechanism for nemertean GVBD, elevated NO/cGMP signaling in SW-stimulated oocytes could deactivate AMPK, as reported for some somatic cells (Lei *et al.* 2005) and thereby promote both MPF activation and maturation (Stricker *et al.* 2010a, Stricker 2011; Fig. 6). Accordingly, cGMP-mediated AMPK deactivation and/or some other SW-stimulated pathway for inhibiting AMPK might co-occur with a separate cAMP-dependent mechanism for downregulating AMPK, such as one involving a PKA-mediated inhibitory phosphorylation at AMPK's Ser 485/491 site (Stricker *et al.* 2010a). To clarify such potential interactions, further analyses are required, including both identifications of possible cGMP targets and time-lapse monitoring of cGMP and cAMP levels.

In other marine invertebrates, cGMP inhibits GVBD in echinoderms and an annelid, which unlike nemerteans use intraoocytic cAMP elevations to prevent GVBD (Nemoto & Ishida 1983, Sato *et al.* 1985, Meijer *et al.* 1989, Karaseva & Khotimchenko 1995). Similarly, even in oocytes of jellyfish and the clam *Spisula* that can undergo cAMP-induced GVBD (Deguchi *et al.* 2011), cGMP is either ineffective or inhibitory to GVBD (Sato *et al.* 1985, Takeda *et al.* 2006). However, unlike in nemerteans, such oocytes do not spontaneously mature in SW. Thus, SW-induced GVBD involving cGMP stimulation might be verified by testing other oocytes that mature in response to both SW and cAMP elevations.

In any case, findings presented here suggest for the first time that SW-induced GVBD can be upregulated by NO/cGMP signaling and that cAMP elevators overcome the inhibitory effects of various NO/cGMP antagonists including AMPK to restore GVBD. Moreover, taking into account the opposite responses to intraoocytic cAMP in nemerteans vs mammals, these results are consistent with previous data showing that, unlike in nemerteans, NO/cGMP signaling blocks GVBD in rats (Nakamura *et al.* 2002, Yamagata *et al.* 2002, Sela-Abramovich *et al.* 2008, Tripathi *et al.* 2009, Pandey *et al.* 2010).

Materials and Methods

Oocyte preparations

Immature oocytes with an intact GV (Stricker & Schatten 1989) were obtained from an undescribed *Cerebratulus* species during summers of 2009 and 2010 on San Juan Island, WA, USA. After removal from gravid worms, oocytes were preincubated 1.5–2 h in ice-cold CaFSW (Schroeder & Stricker 1983) to reduce spontaneous GVBD, which tended to be slightly higher in 2010 than 2009 (data not shown), and preincubated oocytes were dejellied via a Nitex filter to

facilitate pelleting. Dejellied oocytes without follicle cells were then transferred to 24-well culture dishes, with each well containing 1 ml of SW- or CaFSW-solutions at ambient SW temperatures (~12–14 °C).

Experimental solutions and assays

Pharmacological modulators were purchased from AG Scientific (San Diego, CA, USA): DD1, DPTA NONOate, NS2028, ODQ, and Sin-1; Biolog Life Science (Bremen, Germany): 8-Br-cGMP; Cayman Chemicals (Ann Arbor, MI, USA): Sin-1 and YC1; EMD Chemicals (Gibbstown, NJ, USA): isobutylmethylxanthine (IBMX); Enzo Life Sciences (Plymouth Meeting, PA, USA): AICAR, ascorbic acid, CPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt), DD1, DPI chloride, gallotannin, L-NAME (*N*^G-nitro-L-arginine-methyl ester.HCl), NAC; ODQ, PPIX, Sin-1, SNP, tempol, vinpocetine, and zaprinast; LC Labs (Woburn, MA, USA): forskolin; Sigma-Aldrich: 5HT (serotonin), BHA and NAC; Tocris (Ellisville, MO, USA): 8-Br-cGMP and MB.

A few drugs (8-Br-cGMP, ascorbic acid, L-NAME, MB, SNP, and YC1) were dissolved in CaFSW or SW before each test, although even after warming and vigorous vortexing, MB exhibited precipitation in CaFSW/SW solutions and thus failed by an undetermined factor to fully reach its calculated 400 µM dosage level. Alternatively, other drugs were prepared as DMSO- or distilled-water stock solutions, and following dilution, vehicle controls responded like control oocytes lacking the vehicle (data not shown). Working concentrations were based on GVBD dose-response curves or literature reports, which often used higher doses for oocytes than for somatic cells, perhaps because of permeability differences.

Since GVBD typically began by 15–30 min and metaphase-I arrest occurred by 60–90 min, oocytes were continuously incubated with drugs for 1.5–2 h and assayed for GVBD in 50–150 specimens per experimental well by means of an inverted microscope equipped with a 10× objective. Aliquots of counted oocytes were then gently pelleted before removal of excess solution and subsequent freezing in liquid nitrogen (LN). For cAMP elevations, the adenylate cyclase (AC) stimulator forskolin (5 µM) was routinely added, although similar results were also obtained with the PDE inhibitor IBMX (200 µM) or the putative AC stimulator/PDE blocker 5HT (1 µM; data not shown).

Immunoblotting analyses

As described more fully elsewhere (Stricker 2011), cell lysates were obtained for immunoblots from LN-frozen oocytes using lysis buffer containing protease and phosphatase inhibitors. After determining protein content by Bradford assay and boiling samples in Laemmli buffer, 25 µg protein was loaded per lane, run on 4%/10% SDS-PAGE, and transferred onto PVDF membranes. Blots were then treated overnight at 4 °C with primary antibodies (#2535 phospho-T172 AMPK, #9111 phospho-Y15 Cdc2, #9114 phospho-T161 Cdc2, and #9571 phospho-eNOS (Cell Signaling Technology, Danvers, MA, USA)), incubated with HRP-conjugated secondary antibody (Santa Cruz Biochemical, Santa Cruz, CA, USA), visualized

using ECL, and subjected to densitometry of background-subtracted bands.

To assay NO changes during GVBD, aliquots of ~200 oocytes were frozen in LN pre- and poststimulation in SW. After assaying protein content via the Bradford method, samples were then processed for total nitrate/nitrite content using the Griess reagent, according to the manufacturer's directions (Oxford Biochemical, Oxford, MI, USA). Absorptions were normalized relative to total protein values determined for each sample, and NO levels were plotted relative to the prestimulation baseline.

Quantification of data

To minimize the effects of female-specific variability, all experiments utilized oocytes from two or more females. Moreover, at least three replicates ('n' in text and figures) were assessed to obtain the mean $\pm$ S.E.M. values displayed in the text and figures. Statistical analyses involved a Mann–Whitney U- or Student's *t*-test, with significance designated at *P* < 0.05.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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