J Chem Biol (2009) 2:27–37
DOI 10.1007/s12154-008-0013-3

ORIGINAL ARTICLE

Enhancement of the Ca^{2+}-triggering steps of native membrane fusion via thiol-reactivity

Kendra L. Furber · David M. Brandman · Jens R. Coorssen

Received: 13 May 2008 / Accepted: 17 September 2008 / Published online: 1 October 2008

Abstract Ca^{2+}-triggered membrane fusion is the defining step of exocytosis. Isolated urchin cortical vesicles (CV) provide a stage-specific preparation to study the mechanisms by which Ca^{2+} triggers the merger of two apposed native membranes. Thiol-reactive reagents that alkylate free sulfhydryl groups on proteins have been consistently shown to inhibit triggered fusion. Here, we characterize a novel effect of the alkylating reagent iodoacetamide (IA). IA was found to enhance the kinetics and Ca^{2+} sensitivity of both CV-plasma membrane and CV–CV fusion. If Sr^{2+}, a weak Ca^{2+} mimetic, was used to trigger fusion, the potentiation was even greater than that observed for Ca^{2+}, suggesting that IA acts at the Ca^{2+}-sensing step of triggered fusion. Comparison of IA to other reagents indicates that there are at least two distinct thiol sites involved in the underlying fusion mechanism: one that regulates the efficiency of fusion and one that interferes with fusion competency.

Keywords Membrane fusion · Calcium · Strontium · Thiol reactivity · Exocytosis · Vesicle

Abbreviations

CV	Cortical vesicle
PM	Plasma membrane
CSC	Cell surface complex
NEM	*N*-Ethylmaleimide
IA	Iodoacetamide
IAF	Iodoacetamidofluorescein
PDP	Pyridyldithiopropionic acid
AMSDS	4-Acetamido-4′-maleimidylstilbene-2, 2′disulfonic acid

K. L. Furber · D. M. Brandman · J. R. Coorssen
Department of Physiology and Biophysics,
University of Calgary Faculty of Medicine,
Calgary, AB T2N 4N1, Canada

J. R. Coorssen
Department of Biochemistry and Molecular Biology,
University of Calgary Faculty of Medicine,
Calgary, AB T2N 4N1, Canada

J. R. Coorssen
Department of Cell Biology and Anatomy,
University of Calgary Faculty of Medicine,
Calgary, AB T2N 4N1, Canada

K. L. Furber · J. R. Coorssen
Hotchkiss Brain Institute,
University of Calgary Faculty of Medicine,
Calgary, AB T2N 4N1, Canada

J. R. Coorssen (✉)
Molecular Physiology,
School of Medicine, University of Western Sydney,
Bldg. 30,
Campbelltown, NSW 1797, Australia
e-mail: j.coorssen@uws.edu.au

Introduction

Exocytosis is the fundamental process by which eukaryotic cells release specific compounds, including neurotransmitters, hormones, and enzymes. Fast Ca^{2+}-triggered membrane fusion, the defining step of regulated secretion, enables this temporally and spatially specific rapid release process. Yet how Ca^{2+} actually triggers and modulates the merger of the two opposing native membranes remains somewhat speculative.

Urchin cortical vesicles (CV) are a well-established, stage-specific model for the analysis of the molecular

mechanisms underlying fast, Ca^{2+}-triggered native membrane fusion [1–3]. In the intact egg, an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) triggers all the fully docked, primed, and fusion-ready CV to fuse with the plasma membrane (PM); the released content forms the fertilization envelope which prevents polyspermy. Similarly, the cell surface complex (CSC) isolated from the unfertilized egg, consisting of only the fully docked and primed CV attached to fragments of PM, requires no more than an increase in the $[Ca^{2+}]_{free}$ to trigger CV–PM fusion (e.g., exocytosis in vitro) in the absence of other cytosolic factors [4, 5]. The system can be further refined as isolated CV undergo fast, Ca^{2+}-triggered fusion with one another [6–16] and even with pure lipid membranes [12, 17], indicating that CV contain the minimal essential molecular components for intermembrane attachment, Ca^{2+} sensing, and membrane fusion. CV are thus a high purity, robust, stage-specific preparation of release-ready secretory vesicles with which to analyze the mechanisms essential to Ca^{2+}-triggered membrane fusion [6, 7, 14–16].

In earlier efforts to modify the release mechanism, the thiol-reactive reagent, *N*-ethylmaleimide (NEM), was shown to inhibit membrane fusion in both the CV–PM [18–22] and CV–CV assays [6, 11–13]. NEM inhibits both Ca^{2+} sensitivity and the ability to fuse in a dose-dependent manner; treatment with 5 mM NEM consistently leaves CV irreversibly fusion incompetent. Similar patterns of inhibition have also been observed with other thiol-reactive reagents at comparable doses, including sodium tetrathionate [18], pyridyldithiopropionic acid (PDP) [11, 20], and 4-acetamido-4′-maleimidylstilbene-2, 2′disulfonic acid (AMSDS) [11]. One exception is the alkylating reagent iodoacetamide (IA). In earlier studies, a dose of 5 mM IA was shown to have no effect on the extent of either CV–PM [18] or CV–CV fusion (Coorssen, unpublished observation) and was never investigated further.

In contrast to the previous inhibition studies, here we now describe a novel thiol site involved in the enhancement of the Ca^{2+}-triggered membrane fusion mechanism. Substantially higher doses of IA than previously used (10–300 mM) effected a biphasic, dose-dependent potentiation of the Ca^{2+} sensitivity and kinetics of membrane fusion. Further investigation established that IA did not enhance pre-fusion stages; rather, the increase in efficiency was due to modulation of the Ca^{2+}-sensing step of triggered fusion. The differential effects (inhibition vs. potentiation) observed for various thiol-reactive reagents can be attributed to the hydrophobicity of the particular molecule. Overall, the data thus suggest that there are at least two distinct thiol sites that can regulate molecular steps in the triggered fusion pathway.

Materials and methods

Materials The thiol-reactive reagents iodoacetamide, *N*-ethylmaleimide, and maleimide were purchased from Sigma (St. Louis, MO, USA). Flourescein and 5-iodoacetamidofluorescein (IAF) were from Molecular Probes (Eugene, OR, USA). All other chemicals were of at least analytical grade.

CSC and CV preparations CV–PM (cell surface complex; CSC) and CV preparations were isolated from unfertilized sea urchin (*Srongylocentrotus purpuratus*, Westwind, BC, Canada) eggs as previously described [6–9, 14–16]. All treatments and fusion assays were carried out in baseline intracellular medium (BIM; 210 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 10 mM Pipes, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EGTA; pH 6.7; [23]) supplemented with 2.5 mM ATP and protease inhibitors, unless otherwise stated.

Thiol treatments For IA, NEM and maleimide treatments, 2× stock solutions were prepared in BIM and mixed 1:1 with CSC or CV suspensions (OD 1.0–1.2) to the indicated final reagent concentrations. Stock solution of IAF and flourescein (250 mM) were first made in dimethylformamide (DMF) before addition to BIM buffer. Then, CV were suspended directly into BIM containing the fluorescein or IAF solutions (final organic concentration of ≤1%); parallel solvent controls were carried out for these experiments but showed no effect on fusion relative to buffer-only controls (not shown). After incubation with thiol reagents, CSC or CV were washed by centrifugation and suspension in fresh BIM (CSC to OD 0.25–0.30; CV to OD 0.30–0.40) for Ca^{2+}-triggered fusion assays or in Ca^{2+}-free BIM for Sr^{2+}-triggered fusion assays. To assess attachment, a standard chaotrope treatment was used [6–9, 14–16, 24]. Briefly, after IA treatments, each CSC sample was divided into two aliquots and isolated by centrifugation. One aliquot was suspended in BIM for fusion assays and the second suspended in PKME buffer (425 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 50 mM Pipes; pH 6.7; [20]) to release CV from the PM. CSC were incubated in PKME for 1 h at 25°C, and detached CV were subsequently isolated from remaining CSC/PM fragments by a 1-min centrifugation at 700×*g*. The number of free-floating CV in the resulting supernatants was counted using a standard hemocytometer [24].

Fusion assays Standard end-point and kinetic fusion assays were carried out as previously described [6, 7, 9, 14]. For each experiment (*n*), all conditions were tested in replicates

of three to four. Final $[Ca^{2+}]_{free}$ were measured with a Ca^{2+}-sensitive electrode (World Precision Instruments, Sarasota, FL, USA) whereas final free Sr^{2+} concentrations were calculated using MaxChelator (WINMAXC32 v2.50; [25]) as previously described [6]. Fusion data were normalized to the control conditions with Sr^{2+} activity curves normalized to the Ca^{2+} activity curves determined in parallel, as previously described [6, 7, 10]. End-point activity curves were fit using a log-normal cumulative function [26] to determine the extent, slope, and Ca^{2+} sensitivity of fusion; control curves were fit to a two-parameter model (with fusion extent set to 100%, by definition) and data from treated conditions were fit to a three-parameter model using TableCurve 2D (v5.0, SYSTAT, Richmond, CA, USA). For kinetic assays, the initial rapid fusion phase occurring over the duration of the Ca^{2+} injection (450 ms for CV; 720 ms for CSC) is reported as percent fusion per second [14]. Data are reported as mean±SEM; one-way ANOVA, and Bonferroni post hoc analyses were carried out to identify significant differences ($p<0.5$) compared to controls.

Results

IA enhances the Ca^{2+} sensitivity of exocytosis

IA promotes exocytosis (CV–PM fusion) by enhancing the Ca^{2+} sensitivity and rate of fusion without affecting the overall extent of release (Fig. 1). Control CSC fusion was described by a characteristic sigmoidal Ca^{2+} activity curve [6, 14, 26] with an EC_{50} of 55.0±4.4 μM $[Ca^{2+}]_{free}$. Treatment of CSC with moderate doses of IA (60–100 mM) for 20 min caused an increase in the Ca^{2+} sensitivity of exocytosis, measured as a leftward shift in EC_{50} from control values to an average treated value of 23.0±5.7 μM $[Ca^{2+}]_{free}$. Higher doses of IA (160–300 mM) yielded a comparable leftward shift in EC_{50} to 20.5±4.4 μM $[Ca^{2+}]_{free}$ (Fig. 1a,c); moreover, a potentiation of kinetics was also observed following these latter treatments. In response to 73.2±7.1 μM $[Ca^{2+}]_{free}$, the initial rate of fusion increased from a control value of 67.3±4.7% fusion/s to a maximal rate of 87.4±3.6% fusion/s following treatment with 240 mM IA (Fig. 1b,d). There was no significant difference in the extent of fusion with any dose of IA tested (Fig. 1c). Notably, increasing incubation time up to 1 h never resulted in the return of Ca^{2+} sensitivity to control values nor inhibition of the extent of fusion, even with 300 mM IA (data not shown). These results suggest that IA targets thiol group(s) affecting the efficiency of the membrane fusion reaction but that it does not effectively access sites that interfere with the ability of CV to fuse with the target membrane, as is seen with NEM [6, 11–13, 18–22].

To differentiate between possible effects of IA on enhancing the efficacy of docking and thus promoting the Ca^{2+} sensitivity of triggered fusion, as opposed to a more direct effect on the Ca^{2+}-sensing mechanism, we assessed the attachment of CV to the PM using a well-established chaotrope treatment [6–9, 14–16, 24]. CV–PM attachment can be altered by different chaotropic reagents, causing CV to be released from the CSC [24]; indeed, this is the basis for the established CV isolation protocol. Therefore, the number of CV released from the PM during a 1 h incubation in a moderately chaotropic KCl-based buffer [20] was used to evaluate the extent of vesicle attachment (Fig. 2). A 20-min pretreatment with increasing doses of IA had no effect on the number of CV subsequently released from the CSC despite a confirmed increase in the Ca^{2+} sensitivity of exocytosis (e.g., decreased EC_{50}). Thus, IA treatment does not appear to enhance the extent or quality of CV docking to the PM, suggesting that IA promotes the efficiency of the Ca^{2+}-triggering steps of membrane fusion.

IA has a biphasic effect on membrane fusion

To improve the access of reagents to the fusion site and thus better investigate the mechanism underlying the potentiation of fusion, the effect of IA on CV homotypic fusion was assessed. CV were isolated from the PM, treated as free-floating vesicles, washed, and then brought into contact by centrifugation before the addition of increasing $[Ca^{2+}]_{free}$. Consistent with results from the CSC analyses, treatment with IA enhanced both the Ca^{2+} sensitivity and rate of fusion; however, lower doses were required to obtain comparable promotion effects (Fig. 3). Moderate doses of IA (10–80 mM) caused an increase in Ca^{2+} sensitivity from a control EC_{50} of 27.8±1.9 μM to average leftward shifted EC_{50} of 15.0±1.9 μM $[Ca^{2+}]_{free}$ (Fig. 3a,c). IA also potentiated fusion kinetics, increasing the initial rate of fusion in response to 34.8±1.5 μM $[Ca^{2+}]_{free}$ from a control rate of 39.7±5.0% fusion/s to a maximal rate of 84.9±7.8% fusion/s following treatment with 60 mM IA (Fig. 3b,d). Treatment with higher doses of IA (100–300 mM) resulted in the dose-dependent decrease of Ca^{2+} sensitivity that shifted rightward, back to control values (Fig. 3a,c). There was no effect on the extent of fusion except with 80 mM IA. Thiol-reactive reagents can cause nonspecific clumping effects which sometimes caused more CV to be recruited to the ‘lawns’ at the bottom of the multiwell plates (unpublished observation); this occa-

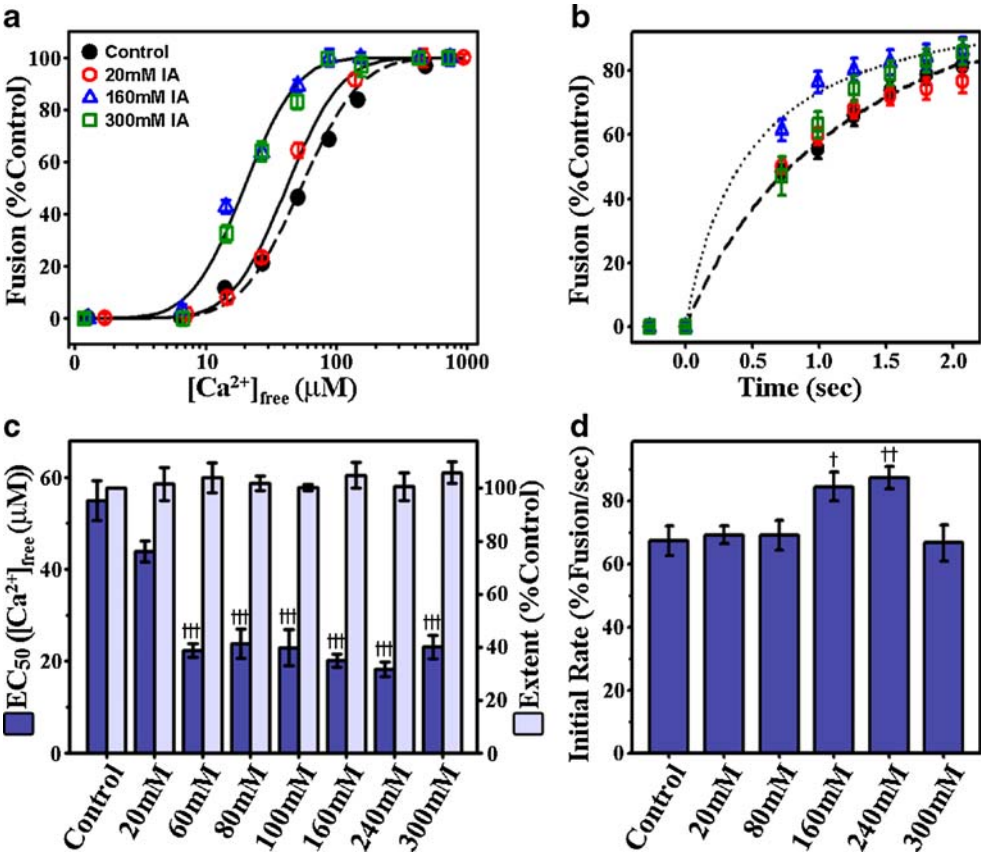

Fig. 1 IA potentiates Ca^{2+} sensitivity and kinetics of exocytotic release as assayed with CSC preparations. **a** Average Ca^{2+} activity curves and **b** average kinetic fusion curves in response to 73±7 μM $[Ca^{2+}]_{free}$ following treatment with representative concentrations of IA for 20 min (control *black circle*, 20 mM *red open circle*, 160 mM *blue open triangle*, 300 mM *green open square*). **c** Summary of average Ca^{2+} activity curve parameters — EC_{50} and extent (n=4–11) and **d** summary of initial rates of fusion (n=7–8) for all concentrations of IA tested. $^{\dagger}p<0.05$ $^{\dagger\dagger}p<0.01$ $^{\dagger\dagger\dagger}p<0.001$

sionally manifests as an apparent increase in fusion extent in some experiments. Due to the high doses of IA required to effect promotion of the fusion parameters, acetamide and *N*-methyl–acetamide were also used to test for potential nonspecific effects that might arise from such high concentrations of IA. Treatments with 80 and 300 mM of these structurally related reagents caused no significant shift in Ca^{2+} sensitivity, initial rate, or extent of fusion (data not shown). Only when the incubation time with IA was increased to 1 h were inhibition of Ca^{2+} sensitivity, kinetics, and extent of fusion observed (see next section, Fig. 6). Similar to CV–PM fusion (Fig. 1), promotion of CV–CV fusion occurred over a broad range of IA doses; however, with isolated CV allowing easy access to the entire vesicle surface, it would appear that the higher doses of IA begin to access those thiol sites that have previously been associated with the inhibition of membrane fusion [18, 20].

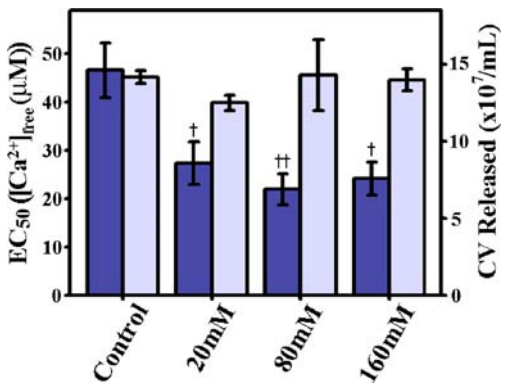

Fig. 2 IA potentiates Ca^{2+} sensitivity of exocytosis but does not affect the quality of CV attachment/docking to the PM. Treatment with increasing concentrations of IA decreased the EC_{50} of the Ca^{2+} activity curves in CSC without altering the number of CV released from the PM by a standard chaotrope treatment (n=2–3). $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$

For CV homotypic fusion, docking/attachment can be assessed using a modified fusion assay [6]. Rather than the standard fusion assay in which CV are centrifuged into contact to most fully enable the docking steps, here CV were allowed simply to settle into contact; as CV contain all necessary components for intermembrane attachment, they still undergo robust fusion in the settle assay. The control EC_{50} in the settle assay was 36.6± 3.6 μM $[Ca^{2+}]_{free}$, and an analogous dose-dependent pattern of increased Ca^{2+} sensitivity was observed following IA treatment (Fig. 4). Moderate doses of IA (10–

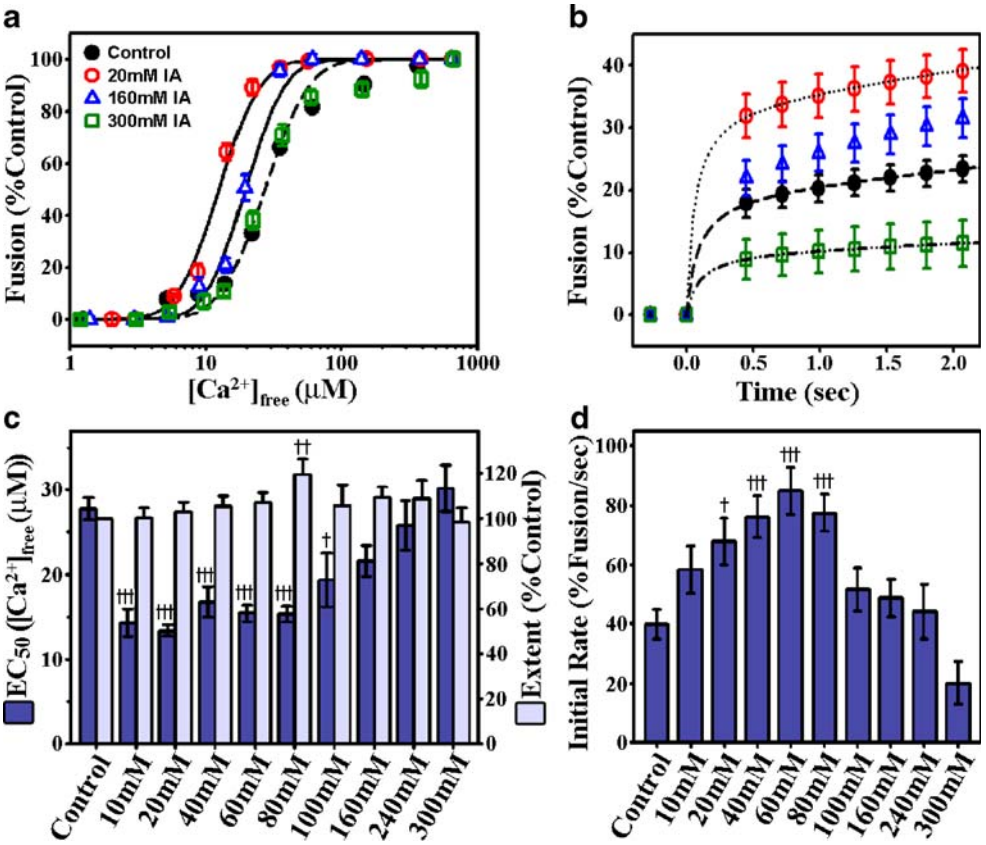

Fig. 3 Biphasic effect of IA on Ca^{2+}-sensitivity and kinetics of CV–CV fusion. **a** Average Ca^{2+} activity curves and **b** average kinetic fusion curves in response to 34.8±1.5 uM $[Ca^{2+}]_{free}$ following exposure to representative concentrations of IA for 20 min (*control*—black circle, 20 mM—*red open circle*, 160—mM *blue open triangle*, 300 mM—*green open square*). **c** Summary of average Ca^{2+} fusion curve parameters (n=5–21), and **d** summary of initial rates of fusion (n=6–15) for all concentrations of IA tested. $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$

80 mM) resulted in an average leftward shift in EC_{50} to 22.0±2.2 μM $[Ca^{2+}]_{free}$, quite consistent with the enhancement seen in the standard CV–CV fusion assay (Fig. 3). Again, there was also no significant effect on the extent of fusion for any of the concentrations of IA tested (Fig. 4b). The equivalent effect of IA in both the settle and standard fusion assays indicates that the enhanced efficiency of fusion does not depend on alterations in vesicle–vesicle attachment.

To further characterize the effect of IA on promoting triggered membrane fusion, we capitalized on the documented ability of Sr^{2+} to substitute for Ca^{2+} [6]. We hypothesized that if IA were promoting Ca^{2+} sensitivity, it might have an even more efficacious effect on the weak triggering seen with Sr^{2+}; indeed, this was the case (Fig. 5). CV–CV fusion triggered with Sr^{2+} yielded a typical sigmoidal but rightward-shifted activity curve relative to parallel Ca^{2+} controls. The EC_{50} was 2.6± 0.6 mM $[Sr^{2+}]_{free}$, and fusion proceeded to the same final extent as seen with Ca^{2+}. As with Ca^{2+} (Fig. 3), moderate doses of IA (20 and 80 mM) produced a maximal leftward shift in EC_{50} to 0.6±0.1 mM $[Sr^{2+}]_{free}$. Higher doses of IA (160 mM and 240 mM) also enhanced the Sr^{2+} sensitivity, which remained unaffected relative to control values only after treatment with 300 mM IA (Fig. 5a,c). In response to 5.5 mM $[Sr^{2+}]_{free}$, potentiation of fusion kinetics was observed from a control rate of 110.4±7.8% fusion/s to a maximal rate of 168.2±12.7% fusion/s after treatment with 80 mM IA, whereas an inhibition of kinetics to 49.9± 4.1% fusion/s was only observed subsequent to treatment with 300 mM IA (Fig. 5b,d). Similar effects of IA were also observed when Sr^{2+} was used to trigger CV–PM fusion, including an enhancement of both Sr^{2+} sensitivity and fusion kinetics (data not shown).

Differential effects of thiol reagents depend on their structure

To better evaluate the ability of IA to access the thiol site(s) that inhibit CV homotypic fusion, incubation times were increased to 1 h, consistent with previous reports using other thiol reagents [6, 11]. Control Ca^{2+} activity curves after this 1 h incubation time had an EC_{50} of 25.0±2.4 μM $[Ca^{2+}]_{free}$ (Fig. 6) and an initial rate of 70.4±5.9% fusion/s in response to 114.5±11.6 μM $[Ca^{2+}]_{free}$ (data not shown), which is comparable to the 20-min incubation times. Even with this prolonged exposure time to IA, significant inhibition of fusion was not observed except at much higher doses. Treatment with 240 mM IA decreased the initial rate to 39.0±9.1% fusion/s (data not shown) and extent of fusion to 70.8±6.5% (Fig. 6c), whereas 300 mM IA decreased the initial rate to 14.8±8.7% fusion/s

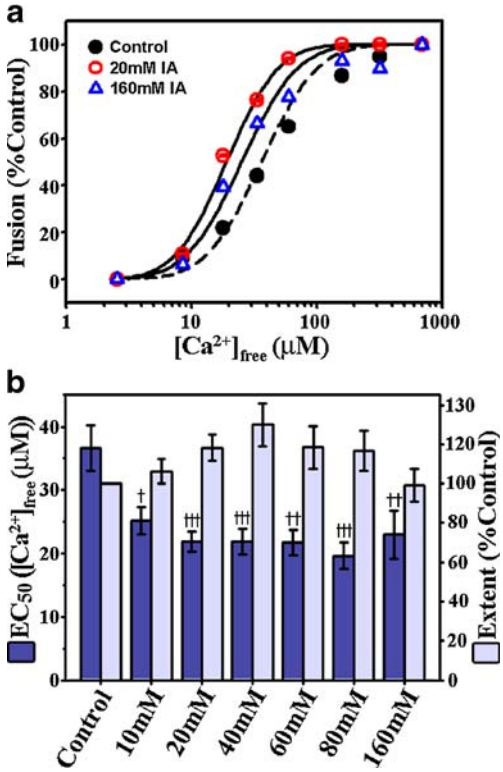

Fig. 4 IA also enhances the Ca^{2+} sensitivity of triggered fusion in a modified settle assay which is used to evaluate the efficacy of intervesicle attachment. **a** Average Ca^{2+} activity curves following exposure to representative concentrations of IA for 20 min (control—*black circle*, 20 mM—*red open circle*; 160 mM—*blue open triangle*) and (**b**) summary of average fusion curve parameters for various doses of IA (n=4–6). $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$

(data not shown), Ca^{2+} sensitivity to 79.3±9.8 μM $[Ca^{2+}]_{free}$, and extent of fusion to 42.3±2.6% (Fig. 6a,c). In comparison, treatment with maleimide and NEM inhibited fusion in the low millimolar range. Treatment with 2.5 mM maleimide inhibited the initial rate to −3.5±5.2% fusion/s (data not shown) and shifted Ca^{2+} sensitivity to 40.3±2.6 μM $[Ca^{2+}]_{free}$ (Fig. 6b), while 10 mM maleimide inhibited the initial rate to 10.6±9.5% fusion/s (data not shown), Ca^{2+} sensitivity to 80.6±7.0 μM $[Ca^{2+}]_{free}$, and extent of fusion to 17.0±9.5% (Fig. 6a,b). NEM was more potent with a 5 mM treatment decreasing the initial rate to 10.8±9.4% fusion/s (data not shown), Ca^{2+} sensitivity to 164.4±12.3 μM $[Ca^{2+}]_{free}$, and extent to 14.5±1.8% (Fig. 6a,b). Notably, if IA is modified with fluorescein (iodoacetamidofluorescein, IAF), the effects on membrane fusion become comparable to those observed with maleimide and NEM, showing a progressive, dose-dependent decrease in kinetics, Ca^{2+} sensitivity, and extent of fusion at doses as low as 1 mM. Following treatment with 2.5 mM IAF, the initial rate decreased to 15.2±3.6% fusion/s (data not shown), Ca^{2+} sensitivity to 105.1±9.8 μM $[Ca^{2+}]_{free}$, and extent to 45.1±7.0% (Fig. 6a,c); however, this reagent could not be tested at higher doses due to solubility limitations. Parallel solvent- and fluorescein-only controls had no effect on Ca^{2+} sensitivity or extent of fusion, indicating that it is the thiol reactivity of IAF that interferes with the fusion mechanism. Furthermore, the presence of solvent did not alter the effects of IA (data not shown). Maleimide, NEM, and IAF were also tested at lower concentrations ranging from 2 μM to 2 mM for short incubation periods (20 min), but none of these treatments resulted in the enhancement of fusion as seen with IA (data not shown). Together, these data indicate that IA (1) preferentially interacts with a novel thiol site(s), not readily accessed by other reagents, to enhance the efficacy of the fusion mechanism and (2) does not react as efficiently as do other reagents with thiol site(s) that inhibit fusion. Figure 6d illustrates the structural differences between IA and these other reagents.

Conclusion

Here we identify a novel effect of thiol-reactive reagents on Ca^{2+}-triggered membrane fusion. Using the stage-specific urchin CSC and CV preparations, previous studies have shown that thiol-reactive reagents inhibit fusion by blocking free sulfhydryl group(s) on proteins [6, 11–13, 18–22]. In contrast, we now show that treatment with the thiol reagent IA has a biphasic effect on membrane fusion. The potentiation of Ca^{2+} sensitivity and kinetics was not due to enhanced efficacy of docking/intermembrane attachment. This novel potentiating effect of IA thus appears linked to the modification of thiol sites that regulate the efficiency of the Ca^{2+}-sensing/tiggering steps of the membrane fusion mechanism. Treatment of CSC with a broad range of IA doses resulted in potentiation of the Ca^{2+} sensitivity and kinetics of fusion. In order to ensure full access of the reagent to the fusion site, so as to analyze this novel promotion of fusion in more detail, we also studied CV isolated from their docked state at the PM. Using dose–response analyses of CV–CV fusion, we were able to differentiate two effects: moderate doses of IA (10–100 mM) for short incubation periods (20 min) potentiated Ca^{2+} sensitivity and kinetics, whereas treatment with high doses (≥240 mM) for longer incubation times (1 h), or with fluorescein-modified IA (1–2.5 mM), inhibited the Ca^{2+} sensitivity, kinetics, and extent of fusion (as seen with other thiol-reactive reagents).

To significantly inhibit fusion, the concentration of IA needed was approximately tenfold that of maleimide and NEM; even this excess amount of IA was still less efficient at inhibiting the kinetics, Ca^{2+} sensitivity, and extent of CV fusion. On the other hand, IAF inhibited fusion at comparable doses to maleimide and NEM. Although the

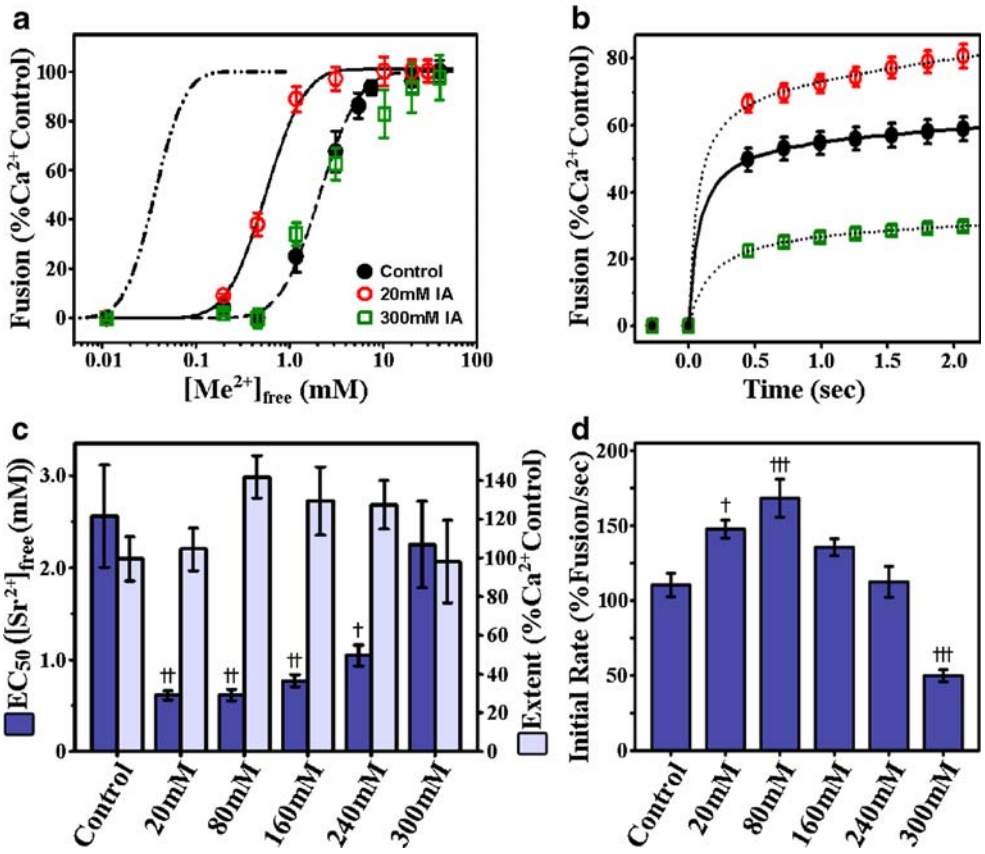

Fig. 5 Biphasic effect of IA on Sr^{2+}-triggered CV–CV fusion. **a** Average Sr^{2+} activity curves following treatment with increasing concentrations of IA (average Ca^{2+} control curve is presented as a *dashed line on left*, control—*black circle*, 20 mM—*red open circle* 300 mM *green open square*). **b** Average fusion kinetics in response to 5.5 mM $[Sr^{2+}]_{free}$ following incubations with specified concentrations of IA (as indicated above). **c** Summary of average Sr^{2+} activity curve parameters (n=3), and **d** summary of initial fusion rates (n=3) for all concentrations of IA tested. $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$

alkylation of cysteine residues was not directly assessed, as treatments were carried out at near-neutral pH it is unlikely that the effects observed were due to nonspecific IA reactions with other amino acid residues [27]. All of the reagents used are considered to be highly selective for thiol groups, which has been previously demonstrated for NEM in the CV model system [18, 20]. Furthermore, the potentiation with IA and the inhibition with IAF are clearly due to the thiol reactivity of the reagents as neither the solvent used (DMF), fluorescein alone, nor structurally related molecules (acetamide and *N*-methylacetamide) had any significant effect on fusion, nor did presence of the solvent alter the effects of IA itself. The differential effects of IAF relative to those of IA, and the similar effects of IAF to maleimide and NEM, indicate that the nature of the alkylation reaction itself does not determine the ability of the reagent to access thiol site(s) critical to membrane fusion. Previous reports have suggested that the potency of the thiol-reactive reagent was dependent on its size [20]. This earlier study used PDP conjugated to large soluble dextrans; however, considering the size of these dextrans and potential cross-linking, a general steric hindrance of attachment and fusion might also result. The size of the reagents used in this study are all relatively small (125–515 g/mol), such that any potential size-related effects should be negligible. Regardless, in terms of size, IAF > IA > NEM > maleimide, whereas the ability of the reagents to inhibit fusion was IAF > NEM > maleimide > IA, clearly indicating that it is not the size of the molecule that correlates with inhibitory potential. Rather, it appears to be the structure of the reagent that is important. The structures of maleimide, NEM, and IAF are all very similar, containing hydrophobic rings that tend to intercalate into membranes [28], whereas IA is a highly polar molecule. The differential effects of these alkylating reagents on CV–CV fusion suggest that the ability to interact with specific thiol site(s), and thus inhibit membrane fusion, is likely to depend on the hydrophobicity of a particular molecule. Similar dependencies have been shown for the thiol modification of the enzyme fumarase; the ability of NEM to interact with cysteine residues buried within the hydrophobic interior of the protein was much greater than that of IA [29]. In the case of membrane fusion, the data here suggest that hydrophobicity is associated positively with the effectiveness for inhibiting fusion and negatively with the capacity to potentiate fusion. These data suggest that there may be at least two

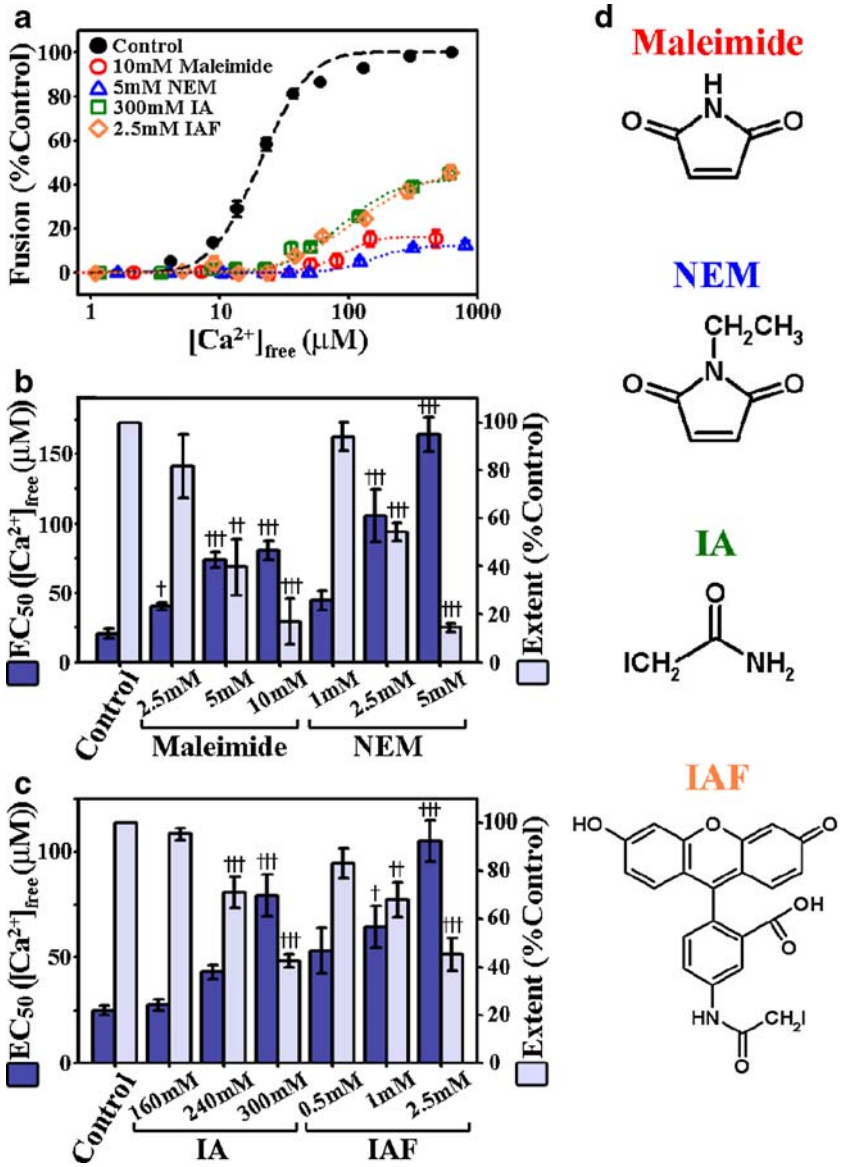

Fig. 6 Comparison of the fusion inhibitory properties of different thiol-reactive reagents in the standard CV–CV fusion assay. **a** Average Ca^{2+} activity curves for indicated doses of maleimide, NEM, IA, and IAF (*black circle*—control, 10 mM maleimide—*red open circle*; 5 mM NEM—*blue open triangle*; 300 mM IA—*green open square*; 2.5 mM IAF—*orange open diamond*). Summary of average fusion curve parameters for various doses of the maleimide- (**b**) and iodacetamide-based (**c**) alkylating reagents (n=4–9). The structure of each reagent is shown in **d**. $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$ $^{\dagger\dagger\dagger}p<0.001$

separate thiol sites implicated in the Ca^{2+}-triggered steps of membrane fusion: (1) a peripheral site involved in the regulation of fusion efficiency (i.e., Ca^{2+} sensitivity and kinetics) and (2) a site closer to or in the membrane (possibly even slightly embedded or buried within the protein) that is more directly involved in the fusion mechanism itself (i.e., ability to fuse).

A variety of assays were used to confirm that the alkylation of thiol sites by IA regulates the efficiency of Ca^{2+}-triggered fusion itself, rather than by enhancing upstream stages of exocytosis. The obvious other possibility was that the treatments enhanced the efficacy of docking, reflected as a more efficient fusion response to a given dose of $[Ca^{2+}]_{free}$. To directly assess intermembrane attachment or docking in CSC preparations, the well-characterized ability of chaotropes to release CV from the PM was employed [24]. Pretreatment with IA did not alter the number of CV released during a subsequent incubation in a chaotropic buffer (Fig. 2). This indicated that treatments with IA did not alter the quality or extent of CV–PM attachment. This was also confirmed for CV–CV attachment. Using a modified fusion assay in which CV are allowed to simply settle into contact enables the assessment of both docking and fusion; in the standard CV–CV fusion assay, CV are centrifuged into contact so as to normalize all samples to the same optimal attached/docked state, thus permitting direct assessment of the Ca^{2+}-triggered fusion steps. In the settle assay, doses of 10–80 mM IA produced

an average leftward shift of 14.6±4.2 μM $[Ca^{2+}]_{free}$ (Fig. 4) which does not differ from the average leftward shift in the EC_{50} of 12.8±2.3 μM $[Ca^{2+}]_{free}$ observed for the same doses of IA when tested in the standard CV fusion assay (Fig. 3). If the enhancement of Ca^{2+} sensitivity was an indirect effect due to an improvement in the upstream stages of the exocytotic pathway, one would expect to see a much more substantial potentiation of fusion in the settle assay. The comparable effect of IA in both assays indicates that this reagent is more likely to affect regulation of the Ca^{2+}-sensing/triggering phase of membrane fusion, rather than enhancing prefusion stages.

In CSC, IA treatment did not cause an increase in the extent of fusion (Fig. 1) which is consistent with previous observations by Vogel et al. [22] that under control conditions >99.9% of CV fuse with the PM in response to ~1 mM $[Ca^{2+}]_{free}$. However, in the CV–CV fusion assays, 80 mM IA did appear to increase the extent of fusion (Fig. 3). Several observations indicate that this is due to a nonspecific clumping effect of thiol-reactive reagents (i.e., an artifact). First, nonspecific clumping can result in an increase in the number of contacting CV. If this is the case, a larger increase in extent would be expected in the settle assay as clumping would facilitate a greater settling of CV to the bottom of the microwell plate. Indeed, in the settle assay, there was a greater tendency for IA to seemingly increase the extent of fusion, although this was never found to be significant. Second, isolated CV remain fully primed and release-ready, requiring only an increase in the $[Ca^{2+}]_{free}$ to trigger fusion (e.g., ATP not required), and fusion appears to proceed via the same common pathway as CV–PM fusion [6, 13]. If an increase in the extent of fusion was due directly to the alkylation of free sulfhydryls then it should occur in both CSC and CV, yet it was observed at only one dose of IA and only with isolated CV. Third, the apparent increase in the extent of fusion following treatment did not correspond to the pattern of enhancement in the efficiency of the fusion mechanism (e.g., Ca^{2+} sensitivity and fusion kinetics) and is thus more consistent with a nonspecific effect. Thus, slight clumping of CV during treatment with this reagent results in a higher number of CV contributing to the fusion signal relative to the untreated controls, yielding the artifact of an apparent increase in the extent of fusion. Why this artifact yielded a statistically significant effect only in the treatments involving 80 mM IA is unknown but not deemed worthy of further pursuit.

Although Sr^{2+} can trigger fusion, it is a relatively inefficient Ca^{2+} mimetic, such that a 100-fold greater concentration of Sr^{2+} (mM) relative to Ca^{2+} (μM) is required [6]. Here we report similar cation dependencies; control fusion curves had an EC_{50} of 27.8±1.9 μM $[Ca^{2+}]_{free}$ and 2.6±0.6 mM $[Sr^{2+}]_{free}$. Nonetheless, when Sr^{2+} was used to trigger fusion, the dose-dependent pattern of potentiation seen following IA treatments was analogous to that seen for Ca^{2+}-triggered fusion. Interestingly, the IA effect appeared somewhat more potent when Sr^{2+} was the trigger for fusion. Doses of 20 and 80 mM IA resulted in an average leftward shift in the EC_{50} of 1.95±0.56 mM $[Sr^{2+}]_{free}$ (Fig. 4) compared to an average leftward shift in the EC_{50} of 14.6±4.2 μM $[Ca^{2+}]_{free}$ (Fig. 3) in the CV–CV fusion assay. When expressed in pMetal units, the increase in Sr^{2+} sensitivity (0.61±0.13 pSr) is approximately twofold greater than the increase in Ca^{2+} sensitivity (0.27±0.06 pCa). Notably, although still higher than the EC_{50} for Ca^{2+}, this maximal leftward shift in EC_{50} to ~600 μM $[Sr^{2+}]_{free}$ brings the triggering effect of this metal into a physiologically relevant range. Thus, this substantial effect on Sr^{2+} sensitivity further reinforces the idea that IA promotes fusion by acting at the level of Ca^{2+} sensing.

The mechanism by which the alkylation of free sulfhydryls with thiol-reactive reagents potentiates Ca^{2+}-triggered membrane fusion is unclear. One possibility is that it acts directly on the Ca^{2+} sensor(s). The oxidation states of sulfhydryls have been shown to affect the affinity of the Ca^{2+}-binding proteins calbindin [30] and calcyclin [31], with the reduced or alkylated form exhibiting enhanced Ca^{2+} binding. Similarly, the modification of thiol groups with alkylating reagents has been shown to affect the affinity of both the IP3 receptor channel [32] and the cGMP channel [33] to their respective ligands; in both cases, there was a biphasic effect of the reagents such that lower doses increased and higher doses decreased the apparent affinity for the ligand depending on which thiol sites were affected. With respect to the data presented here, the simplest model would involve a single Ca^{2+} sensor with two distinct thiol sites: one that regulates Ca^{2+} sensitivity and one that is critical to its ability to trigger fusion.

However, one must consider the emerging view that the fusion complex is potentially comprised of several proteins that work in concert with specific lipids and with each other. Thus, inhibitory effects could be related to a specific protein suffering altered interactions with a neighboring protein, altered interactions with lipids, or both. In terms of potentiating Ca^{2+} sensitivity, one interpretation is simply that the binding affinity of a critical Ca^{2+} sensor is enhanced, but the results with Sr^{2+} do suggest an alternate possibility. Weak Ca^{2+} mimetics such as Sr^{2+} are capable of interacting with a specific Ca^{2+} sensor that interacts directly with, and thus triggers, the fusion machinery; in contrast though, the Sr^{2+} ions bind poorly to alternate sights or even alternate sensors that modulate the efficiency of the fusion mechanism [6]. Therefore, IA may not necessarily act on a Ca^{2+} sensor (e.g., "trigger") required for fusion per se but may modify secondary Ca^{2+} sensors that mediate the efficiency of the fusion mechanism. Furthermore, we

cannot yet exclude the fact that IA may act on a protein that regulates a Ca^{2+} sensor. Recently, several chaperones have been implicated in maintaining effective protein conformations or mediating the interactions of several exocytotic proteins [34–36]. Such a regulatory protein might even act through a negative clamp-like mechanism, such that blocking a disulfide interaction relieves inhibition of the Ca^{2+} sensor.

Delineating the nature of the targeted thiol effect will require the definitive identification of the protein complex involved in the docking and fusion mechanisms. Using the thiol-reactive reagent IA, one can alter the mechanism by either potentiating or inhibiting Ca^{2+}-triggered fusion. This indicates that there are at least two thiol sites involved in the underlying mechanism. Thorough characterization of the potentiation effect indicates that the relevant thiol site(s) are on proteins functioning at the level of Ca^{2+} sensing, whereas the alternate thiol site is associated with proteins critical to fusion competency. Nonetheless, it is still unclear whether these cysteine residues belong to a single protein or protein complex. The use of thiol-reactive reagents having differing attributes should provide additional tools to explore these two opposing effects and promise to further promote our understanding of the molecular machinery underlying Ca^{2+}-triggered membrane fusion.

Acknowledgements J.R.C. acknowledges support from CIHR, NSERC, and AHFMR. K.L.F acknowledges support from CHIR and AHFMR, and D.M.B. from NSERC. The authors wish to thank R.H. Butt, M.A. Churchward, T. Rogasevskaia, M. Ernst, and F. Rhemtulla for helpful disscussions, and C. Skolseg and D. Bininda for assistance with aquatics.

References

1. Zimmerberg J, Coorssen JR, Vogel SS, Blank PS (1999) Sea urchin egg preparations as systems for the study of calcium-triggered exocytosis. J Physiol 520(Pt 1):15–21
2. Zimmerberg J, Blank PS, Kolosova I, Cho MS, Tahara M, Coorssen JR (2000) A stage-specific preparation to study the Ca(2+)-triggered fusion steps of exocytosis: rationale and perspectives. Biochimie 82:303–314
3. Szule JA, Coorssen JR (2003) Revisiting the role of SNAREs in exocytosis and membrane fusion. Biochim Biophys Acta 1641:121–135
4. Baker PF, Whitaker MJ (1978) Influence of ATP and calcium on the cortical reaction in sea urchin eggs. Nature 276:513–515
5. Vacquier VD (1975) The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. Dev Biol 43:62–74
6. Coorssen JR, Blank PS, Tahara M, Zimmerberg J (1998) Biochemical and functional studies of cortical vesicle fusion: the SNARE complex and Ca^{2+} sensitivity. J Cell Biol 143:1845–1857
7. Coorssen JR, Blank PS, Albertorio F, Bezrukov L, Kolosova I, Chen X, Backlund PS Jr, Zimmerberg J (2003) Regulated secretion: SNARE density, vesicle fusion and calcium dependence. J Cell Sci 116:2087–2097
8. Hibbert JE, Butt RH, Coorssen JR (2006) Actin is not an essential component in the mechanism of calcium-triggered vesicle fusion. Int J Biochem Cell Biol 38:461–471
9. Szule JA, Jarvis SE, Hibbert JE, Spafford JD, Braun JE, Zamponi GW, Wessel GM, Coorssen JR (2003) Calcium-triggered membrane fusion proceeds independently of specific presynaptic proteins. J Biol Chem 278:24251–24254
10. Tahara M, Coorssen JR, Timmers K, Blank PS, Whalley T, Scheller R, Zimmerberg J (1998) Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. J Biol Chem 273:33667–33673
11. Whalley T, Timmers K, Coorssen J, Bezrukov L, Kingsley DH, Zimmerberg J (2004) Membrane fusion of secretory vesicles of the sea urchin egg in the absence of NSF. J Cell Sci 117:2345–2356
12. Vogel SS, Chernomordik LV, Zimmerberg J (1992) Calcium-triggered fusion of exocytotic granules requires proteins in only one membrane. J Biol Chem 267:25640–25643
13. Vogel SS, Zimmerberg J (1992) Proteins on exocytic vesicles mediate calcium-triggered fusion. Proc Natl Acad Sci U S A 89:4749–4753
14. Churchward MA, Rogasevskaia T, Hofgen J, Bau J, Coorssen JR (2005) Cholesterol facilitates the native mechanism of Ca^{2+}-triggered membrane fusion. J Cell Sci 118:4833–4848
15. Rogasevskaia T, Coorssen JR (2006) Sphingomyelin-enriched microdomains define the efficiency of native Ca^{2+}-triggered membrane fusion. J Cell Sci 119:2688–2694
16. Churchward MA, Rogasevskaia T, Brandman DM, Khosravani H, Nava P, Atkinson JK, Coorssen JR (2008) Specific lipids supply critical negative spontaneous curvature—an essential component of native Ca^{2+}-triggered membrane fusion. Biophys J 94:3976–3986
17. Chanturiya A, Whitaker M, Zimmerberg J (1999) Calcium-induced fusion of sea urchin egg secretory vesicles with planar phospholipid bilayer membranes. Mol Membr Biol 16:89–94
18. Haggerty JG, Jackson RC (1983) Release of granule contents from sea urchin egg cortices. New assay procedures and inhibition by sulfhydryl-modifying reagents. J Biol Chem 258:1819–1825
19. Jackson RC, Ward KK, Haggerty JG (1985) Mild proteolytic digestion restores exocytotic activity to *N*-ethylmaleimide-inactivated cell surface complex from sea urchin eggs. J Cell Biol 101:6–11
20. Whalley T, Sokoloff A (1994) The *N*-ethylmaleimide-sensitive protein thiol groups necessary for sea-urchin egg cortical-granule exocytosis are highly exposed to the medium and are required for triggering by Ca^{2+}. Biochem J 302(Pt 2):391–396
21. Jackson RC, Modern PA (1990) *N*-Ethylmaleimide-sensitive protein(s) involved in cortical exocytosis in the sea urchin egg: localization to both cortical vesicles and plasma membrane. J Cell Sci 96(Pt 2):313–321
22. Vogel SS, Blank PS, Zimmerberg J (1996) Poisson-distributed active fusion complexes underlie the control of the rate and extent of exocytosis by calcium. J Cell Biol 134:329–338
23. Coorssen JR, Blank PS, Albertorio F, Bezrukov L, Kolosova I, Backlund PS Jr, Zimmerberg J (2002) Quantitative femto- to attomole immunodetection of regulated secretory vesicle proteins critical to exocytosis. Anal Biochem 307:54–62
24. Decker SJ, Kinsey WH (1983) Characterization of cortical secretory vesicles from the sea urchin egg. Dev Biol 96:37–45
25. Bers DM, Patton CW, Nuccitelli R (1994) A practical guide to the preparation of Ca^{2+} buffers. Methods Cell Biol 40:3–29
26. Blank PS, Cho MS, Vogel SS, Kaplan D, Kang A, Malley J, Zimmerberg J (1998) Submaximal responses in calcium-triggered exocytosis are explained by differences in the calcium sensitivity of individual secretory vesicles. J Gen Physiol 112:559–567

27. Hopkins CE, Hernandez G, Lee JP, Tolan DR (2005) Aminoethylation in model peptides reveals conditions for maximizing thiol specificity. Arch Biochem Biophys 443:1–10
28. McLaughlin S, Whitaker M (1988) Cations that alter surface potentials of lipid bilayers increase the calcium requirement for exocytosis in sea urchin eggs. J Physiol 396:189–204
29. Robinson GW, Bradshaw RA, Kanarek L, Hill RL (1967) The thiol groups of fumarase. J Biol Chem 242:2709–2718
30. Vanbelle C, Halgand F, Cedervall T, Thulin E, Akerfeldt KS, Laprevote O, Linse S (2005) Deamidation and disulfide bridge formation in human calbindin D28k with effects on calcium binding. Protein Sci 14:968–979
31. Kordowska J, Stafford WF, Wang CL (1998) Ca^{2+} and Zn^{2+} bind to different sites and induce different conformational changes in human calcyclin. Eur J Biochem 253:57–66
32. Poirier SN, Poitras M, Laflamme K, Guillemette G (2001) Thiol-reactive agents biphasically regulate inositol 1,4,5-trisphosphate binding and Ca(2+) release activities in bovine adrenal cortex microsomes. Endocrinology 142:2614–2621
33. Serre V, Ildefonse M, Bennett N (1995) Effects of cysteine modification on the activity of the cGMP-gated channel from retinal rods. J Membr Biol 146:145–162
34. Evans GJ, Morgan A, Burgoyne RD (2003) Tying everything together: the multiple roles of cysteine string protein (CSP) in regulated exocytosis. Traffic 4:653–659
35. Zinsmaier KE, Bronk P (2001) Molecular chaperones and the regulation of neurotransmitter exocytosis. Biochem Pharmacol 62:1–11
36. Latham CF, Meunier FA (2007) Munc18a: Munc-y business in mediating exocytosis. Int J Biochem Cell Biol 39:1576–1581