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Triazine dyes are agonists of the NAADP receptor

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1 NAADP has been shown to be a potent calcium-releasing second messenger in a wide variety of cell types to date. However, research has been hampered by a lack of pharmacological agents, with which to investigate NAADP-induced calcium release, and by the molecular identity of its cellular target protein being unknown.

2 In the present paper, the sea urchin egg model was used to investigate whether triazine dyes, which can act as nucleotide mimetics, can bind to the NAADP receptor, induce Ca^{2+} release and be used for affinity chromatography of the receptor.

3 Indeed, all the triazine dyes tested (Reactive Red 120 (RR120), Reactive Green 19 (RG19), Reactive Green 5 (RG5), Cibacron Blue 3GA and Reactive Yellow 86) displayed micromolar affinities, except for Reactive Orange 14. Furthermore, unlike NAADP, RR120, RG19 and RG5 did not bind in an irreversible manner.

4 The compound that displayed the highest affinity, RR120, was tested in a $^{45}Ca^{2+}$ efflux assay. This compound released Ca^{2+} *via* the NAADP receptor, as shown by the ability of subthreshold NAADP concentrations to inhibit this release. Furthermore, heparin and ruthenium red were unable to block RR120-induced Ca^{2+} release.

5 We have also shown that RG5 and RG19, immobilised on resins, retain the ability to bind to the receptor, and that this interaction can be disrupted by high salt concentrations. As a proof of principle, we have shown that this can be used to partially purify the NAADP receptor by at least 75-fold.

6 In conclusion, triazine dyes interact with the NAADP receptor, and this could be exploited in future to create a new generation of pharmacological tools to investigate this messenger and, in combination with other techniques, to purify the receptor.
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Abbreviations: CB, Cibacron Blue 3GA; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; RG5, Reactive Green 5; RG19, Reactive Green 19; RR120, Reactive Red 120

Introduction

Nicotinic acid adenine dinucleotide phosphate (NAADP) has been shown to release Ca^{2+} from intracellular stores in a number of models, ranging from echinoderms to a variety of mammalian cell types (Patel *et al*., 2001; Genazzani & Billington, 2002). Recently, its potential role in cellular responses in these cell types is beginning to be elucidated. For example, in sea urchin eggs, NAADP appears to play a role in the characteristic Ca^{2+} transients at fertilisation (Billington *et al*., 2002; Churchill *et al*., 2003), while in mammalian cells, alongside Ca^{2+} release (see Yamasaki *et al*. (2004) for an example), one of its roles may be to modulate Ca^{2+} signals induced IP_3 and cADPR (Cancela *et al*., 1999; 2002; Berg *et al*., 2000).

Nonetheless, research in this field has been hampered by the lack of pharmacological agents capable of modulating NAADP-induced Ca^{2+} release. Several compounds have been shown to act as agonists of the receptor, but these have all been very close structural analogues of NAADP (Lee & Aarhus, 1997). Further to this, NAADP is able to act as its own antagonist (Aarhus *et al*., 1996; Genazzani *et al*., 1996a). In sea urchin eggs, very low NAADP concentrations, which are unable to cause detectable Ca^{2+} release, completely desensitise the receptor to further challenges of NAADP (Aarhus *et al*., 1996; Genazzani *et al*., 1996a). Conversely, in mammalian cells very high concentrations of NAADP desensitise the receptor without releasing Ca^{2+} (Cancela *et al*., 1999; Berg *et al*., 2000). A number of other pharmacological agents have also been shown to inhibit NAADP-induced Ca^{2+} release, but none of these display specificity (Genazzani *et al*., 1997). For example, it has been shown that high concentrations of L-type Ca^{2+}-channel blockers and K^+-channel blockers abolish NAADP-induced Ca^{2+}

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release in sea urchin egg homogenates, but this occurs only at concentrations above those required to block plasma membrane channels (Genazzani *et al*., 1997). Furthermore, these drugs do not interact with the NAADP-binding site on the receptor (Genazzani *et al*., 1997). In addition to the lack of pharmacological agents that can be used in functional assays, the molecular identity of the NAADP receptor protein/NAADP-sensitive Ca^{2+} channel is, as yet, unknown.

Triazine dyes, developed as commercial textile dyes, have proven invaluable tools for both the study and purification of proteins (Janson & Ryden, 1998; Denzili & Piskin, 2001). Superficially, they show little structural homology to nucleotides; yet they are able to act as nucleotide mimetics with surprisingly high affinities for nucleotide-binding sites (Denzili & Piskin, 2001).

Here we show that a number of triazine dyes show affinity for the NAADP receptor in radioligand-binding assays and that the majority of them are able to bind in a reversible manner. Furthermore, Reactive Red 120 (RR120) is also able to activate the NAADP receptor and act as an agonist in Ca^{2+}-release experiments. Due to the affinity of these compounds for the NAADP receptor and their previous use in affinity chromatography protocols, we have investigated whether immobilised triazine dyes could be used in the purification of the NAADP receptor. We found that both immobilised Reactive Green 19 (RG19) and immobilised Reactive Green 5 (RG5) are able to bind to the receptor and that the protein may be eluted from the beads using high concentrations of salts. This report, therefore, provides evidence for a new class of drugs that act on the NAADP receptor at the NAADP-binding site and might assist in the purification of the receptor protein.

Methods

Sea urchin egg homogenate

Eggs and sperm of *Lytechinus pictus* (Marinus Inc., Long Beach, CA, U.S.A.) were obtained by intracoelomic injection of 0.5 M KCl and collected in Artificial Sea Water (Kent Sea Salt, Kent Marine Inc, Georgia, U.S.A.). Egg homogenates were prepared as described previously (Dargie *et al*., 1990) and stored at −80°C until required.

$^{45}Ca^{2+}$-release assays

$^{45}Ca^{2+}$-release assays were performed as described previously (Bak *et al*., 2002). Homogenates from sea urchin eggs were diluted 40-fold into a Glu-IM containing $^{45}CaCl_2$, 1 mM ATP, 10 mM phosphocreatine and 10 U ml^{-1} creatine phosphokinase. Approximately 50 nCi $^{45}CaCl_2$ was used per assay point. Calcium uptake was performed at room temperature for 15–45 min. Release was initiated by the addition of the appropriate agonist to a homogenate batch. The $^{45}Ca^{2+}$ that remained in the vesicles was determined by filtration of 0.5 ml egg homogenate through a pre-washed nitrocellulose Millipore filter under vacuum. Filters were then rapidly washed twice with 1.5 ml ice-cold IM containing 5 mM $LaCl_3$. The radioactivity retained on the filter was determined using standard liquid scintillation counting.

[^{32}P]NAADP-binding assays

Binding of [^{32}P]NAADP in sea urchin egg homogenates was determined using a centrifugation protocol. Binding experiments were carried out on ice in Intracellular Medium (Glu-IM; 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.2). [^{32}P]NAADP was added at an approximate final concentration of 50 pM (10,000–15,000 c.p.m.) and competing ligand was added to this mixture. The binding reaction was started by addition of sea urchin egg homogenate (50 μg) or solubilised protein. Incubations were incubated on ice for 20 min and terminated by centrifugation at 20,000 × *g* for 5 min at 4°C. The pellet was washed once in 1 ml ice-cold Glu-IM and resuspended in 1 ml scintillation fluid. Binding reactions with solubilised protein were terminated by polyethyleneglycol (PEG) precipitation. Briefly, γ-globulins were added to the binding reaction at a final concentration of 4 mg ml^{-1} and PEG was added to a final concentration of 15% (w v^{-1}). The reaction was vortexed and incubated on ice for 5 min before centrifugation. The reactions then proceeded as above.

Reversible binding experiments were performed as follows; 50 μg homogenate was treated with dye at concentrations above the EC_{50} for 20 min (final volume 40 μl). This was then diluted in 3600 μl IM containing 50 pM [^{32}P]NAADP to decrease the dye concentration to noninhibitory concentrations. Reactions then proceeded as above.

Affinity chromatography

Sea urchin egg microsomes were prepared by a centrifugation protocol. Egg homogenate (50%) was diluted to 12.5% in IM and spun at 10,000 × *g* for 10 min. The resulting supernatant was spun at 100,000 g for 1 h and the pellet (microsomes) retained. Microsomes were solubilised in CHAPS (1% final concentration) for 3 h on ice and then spun at 100,000 × *g* for 1 h. The supernatant containing the solubilised proteins was retained and the insoluble fraction (pellet) discarded.

Immobilised triazine dyes were obtained from SIGMA (Poole, U.K.). RG5, RG19 and RR120 were obtained coupled to agarose beads, while Cibacron Blue 3GA (CB) was obtained coupled to DEAE cellulose beads. Beads were incubated with solubilised protein in a buffer containing 20 mM HEPES, 0.5% CHAPS (pH 7.2), in the presence or absence of varying concentrations of NaCl or $BaCl_2$. After incubation on a rotary mixer for 1 h at 4°C, the reaction was spun briefly at 13,000 × *g* and the supernatant removed. An aliquot of this was taken and radioligand binding was performed as described using this aliquot in place of membranes. The total [^{32}P]NAADP binding in the bead-treated sample was compared to the total binding in an identical sample not treated with beads.

For larger-scale experiments, 2 ml of the dye beads was packed into gravity columns (Poly-Prep; Bio-Rad, U.K.). Columns were washed extensively in 20 mM HEPES, 0.5% CHAPS, pH 7.2, before 1 mg solubilised sea urchin egg membranes were loaded in the same buffer. Washing and elution in salts was performed by dissolving the appropriate concentration of salt in 20 mM HEPES, 0.5% CHAPS, pH 7.2.

Fractions were collected throughout and assayed for both protein content (Bradford assay) and [^{32}P]NAADP binding. High concentrations of NaCl and $BaCl_2$ were found to be inhibitory to the binding of [^{32}P]NAADP to its receptor;

therefore, samples of salt-containing fractions were diluted before addition to the binding reaction to reduce the salt to noninhibitory concentrations.

Results

To assess whether triazine dyes are recognised by the sea urchin NAADP receptor, [^{32}P]NAADP competition-binding assays were performed in the presence of increasing concentrations of commercially available triazine dyes. All the compounds tested, except Reactive Orange 14, were able to compete fully for the NAADP-binding site at concentrations lower than 1 mM (Figure 1). Compounds showed micromolar affinities for the receptor, with RR120 showing the highest affinity (Table 1, Figure 1). Unlike NAADP, which displays a Hill slope of 1 for binding (Billington & Genazzani, 2000), these compounds displayed Hill slopes between 1.9 and 3.8, suggesting that the mode of interaction might not be identical.

It has been shown previously that, *in vitro*, NAADP binds to the sea urchin receptor in an irreversible manner under conditions mimicking the cellular environment (Billington & Genazzani, 2000; Patel *et al*., 2000; Dickinson & Patel, 2003). To investigate whether the triazine dyes displayed a similar property, sea urchin egg homogenates were pretreated for 20 min with concentrations of NAADP or dye that, in competition assays, displaced more than 90% of the [^{32}P]NAADP. Samples were then diluted 100-fold with buffer containing 50 pM [^{32}P]NAADP, and the resultant bound radioligand was determined by rapid filtration. As previously shown, pre-incubation with 1 nM NAADP prevented any further binding of [^{32}P]NAADP to the receptor (Table 2; Billington & Genazzani, 2000). Similarly, pre-incubation of membranes with CB was also able to prevent any further binding of [^{32}P]NAADP, suggesting that this compound binds in an irreversible manner. On the contrary, when samples were pre-incubated with RR120, RG19 or RG5, [^{32}P]NAADP was able to displace the pre-incubated dye and bind to the receptor, showing that these compounds bind in a reversible manner.

Since triazine dyes are capable of binding to the receptor, we next tested whether high concentrations of RR120, the drug that displayed the highest affinity, could elicit Ca^{2+} release from sea urchin egg homogenates. Since these dyes interfere with fluorescent determinations, $^{45}Ca^{2+}$ measurements were performed in actively loaded sea urchin egg microsomes. When microsomes were challenged with either NAADP (2 μM) or RR120 (100 μM), a similar amount of Ca^{2+} release was evoked. It has been previously shown that RR120 and Reactive Blue 2 are capable of activating the ryanodine receptor (Xu *et al*., 1989) and that Reactive Blue 2 is capable of inducing Ca^{2+} oscillations in HeLa cells (Okuda *et al*., 2001). Furthermore, CB and Patent blue have been shown to compete for IP_3 binding in rat liver (Bootman *et al*., 1990) Therefore, further analysis was required to prove whether NAADP and RR120 released Ca^{2+} *via* the same mechanism. RR120-induced Ca^{2+} release was not prevented by treatment with the IP_3 receptor antagonist heparin (600 μg ml^{-1}), or the ryanodine receptor antagonist ruthenium red (5 μM). Indeed, a potentiation of the Ca^{2+} release was observed with either agent (control release 10.1±0.35% of total stored calcium; heparin 26±1.7%; ruthenium red 16.8±1.6%). These data would indicate that RR120-induced Ca^{2+} release is independent of IP_3 or ryanodine receptors, although some functional interaction, probably dependent on the assay, is occurring. To investigate further whether RR120 releases Ca^{2+} *via* the NAADP receptor, receptors were desensitised by pretreating the homogenate with a nonreleasing concentration of NAADP (3 nM), a condition that abolishes further NAADP-induced Ca^{2+} release, as previously reported (Aarhus *et al*., 1996; Genazzani *et al*., 1996a; Figure 2). Under these conditions, RR120-induced Ca^{2+} release was significantly reduced, demonstrating that this drug acts as an agonist at the receptor (Figure 2).

Triazine dyes have been extensively used as affinity probes to partially purify NAD- and NADP-utilising enzymes (Janson & Ryden, 1998; Denzili & Piskin, 2001). Therefore, it was investigated whether immobilised dyes could be used in

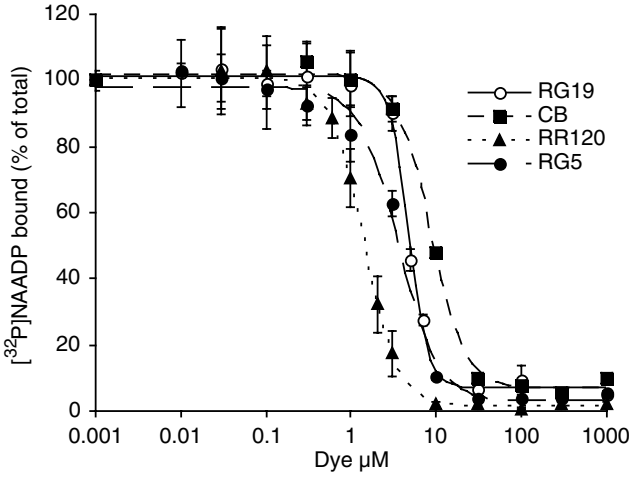

Figure 1 Binding curves for triazine dyes competing with [^{32}P]NAADP in sea urchin egg homogenate. $n = 9$–12.

Table 1 Half-maximal inhibition of [^{32}P]NAADP binding to sea urchin egg homogenate by triazine dyes

Ligand	IC_{50} (μM)
Reactive Red 120	1.43±0.04
Cibacron Blue 3GA	8.56±0.57
Reactive Green 19	3.57±0.36
Reactive Green 5	4.74±0.12
Reactive Yellow 86	58.98±5.43
Reactive Orange 14	>1 mM

Results are expressed as the average±s.e.m. of 4–9 independent determinations.

Table 2 Reversibility of triazine dyes binding to the NAADP receptor

Pretreatment	*Recovery of [^{32}P]NAADP binding (% of control)*
Control	100±2.4
NAADP (1 nM)	14.6±2.9
Reactive Red 120 (30 μM)	65.8±6.9
Reactive Green 5 (30 μM)	89.3±5.4
Reactive Green 19 (30 μM)	86.1±8.5
Cibacron Blue 3GA (100 μM)	18.8±2.4

Results are expressed as the average±s.e.m. of 10–14 independent determinations.

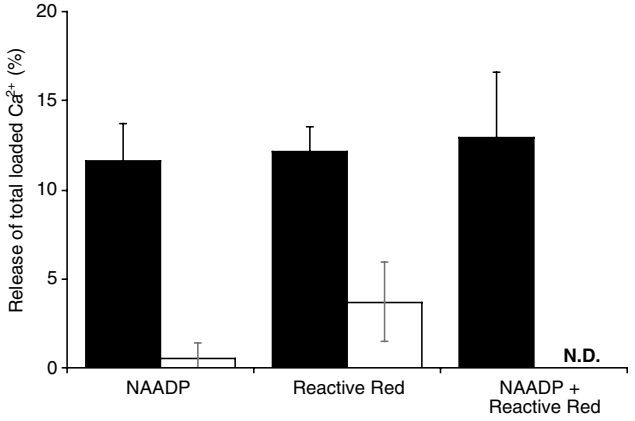

Figure 2 $^{45}Ca^{2+}$ release in response to NAADP (2 μM) and RR120 (100 μM) (filled bars). Open bars show the release following pretreatment with 3 nM NAADP. $n = 9$–14.

order to achieve a partial purification of the receptor. To assess this, pull-down experiments using commercially available dye-coupled beads were performed and residual binding in supernatants was assessed as an index of the quantity of receptor that did not bind to the immobilised compound compared to control. CHAPS-solubilised membranes, which have been previously shown to contain the NAADP receptor (Berridge *et al*., 2002), were used as a source of protein. While CB- and RR120-coupled beads were unable to pull down the receptor reproducibly (data not shown), both RG5 and RG19 bound avidly to the receptor protein. RG5 agarose absorbed $82.5 \pm 1.5\%$ ($n = 9$) of the receptor from the solubilised protein sample, as measured by radioligand binding in treated supernatants, while RG19 agarose absorbed $86.4 \pm 1.4\%$ ($n = 8$) of the receptor. To test whether this method could be used to partially purify the receptor and to characterise elution conditions, we incubated solubilised protein (50–75 μg) with RG5 and RG19 agarose beads in the presence of increasing concentrations of NaCl and measured the amount of receptor that was not absorbed to the beads by radioligand binding. Since NaCl has been shown to affect NAADP binding to its receptor (Patel *et al*., 2000), the supernatants with the unbound receptor were diluted such that the salt concentration was reduced to noneffective concentrations (100 mM). NaCl inhibited binding of the receptor to the RG19 beads in a concentration-dependent manner (IC_{50} approx. 500 mM, Figure 3a), while concentrations of NaCl up to 1.5 M were unable to significantly affect the binding of the receptor to RG5 beads. Nonetheless, $BaCl_2$ was capable of inhibiting the binding of the receptor to the beads. In principle, therefore, it could be possible to wash the RG5 beads with high concentrations of NaCl in order to remove weakly bound proteins and to elute the receptor with $BaCl_2$. This would be of importance in a purification procedure since it has been shown that these molecules are capable of binding to a number of proteins. To test whether this strategy could work in practice, 1 mg of solubilised protein was loaded in a gravity-packed RG5 column and the column was washed with 4 column volumes of 1.5 M NaCl (in 20 mM HEPES, 0.5% CHAPS). The flow-through and the fractions eluting with NaCl contained the majority of the protein content, while [^{32}P]NAADP binding was close to the nonspecific levels (Figure 3c). When elution was carried out sequentially in 0.6 M $BaCl_2$, the

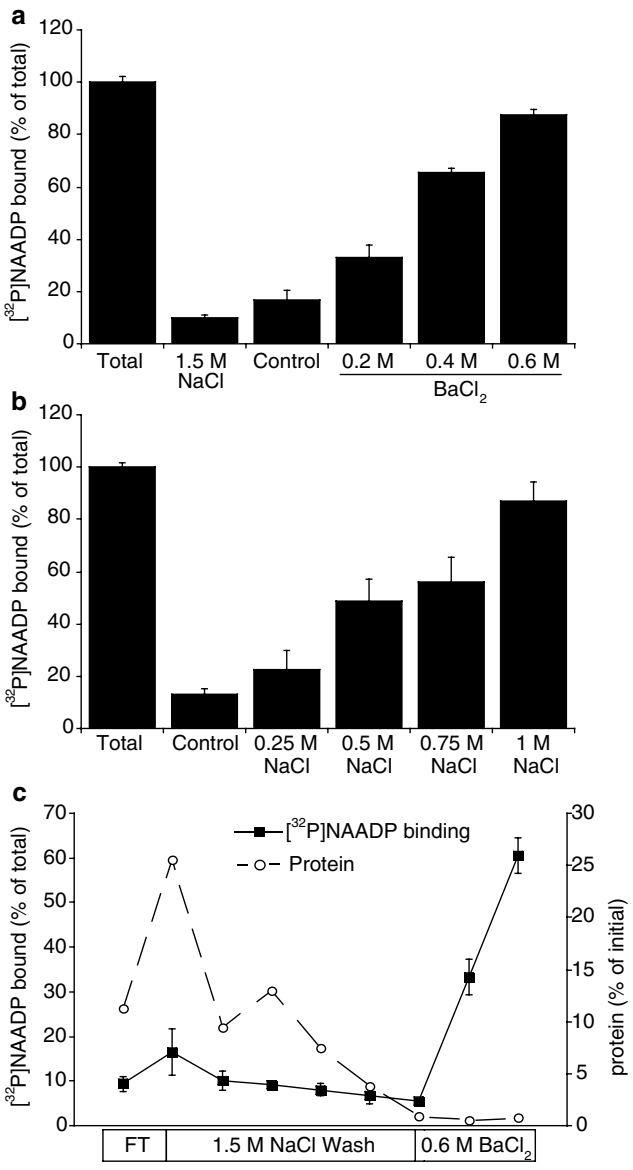

Figure 3 (a) Inhibition of NAADP receptor binding to RG19 agarose by increasing concentrations of NaCl. $n = 6$–9. (b) Inhibition of NAADP receptor binding to RG5 agarose by $BaCl_2$ and NaCl. $n = 8$–9. (c) Partial purification of the NAADP receptor using a RG5 agarose column. The relative protein content of each fraction (% of initial 1 mg solubilised protein applied to column) was measured by Bradford assay. Specific [^{32}P]NAADP binding detected in each fraction is expressed as the % of total [^{32}P]NAADP binding detected in 1 mg solubilised protein. $n = 6$.

majority of the receptor, as detected by [^{32}P]NAADP binding, was found in the first two washes (Figure 3c), while further washes did not yield significant receptor protein. Such a one-step procedure increased the concentration of the receptor approximately 75-fold compared to the initial solubilised protein (B_{max}: solubilised protein 280 fmol mg^{-1}; eluent 21.2 pmol mg^{-1}).

Discussion

Triazine dyes have been used widely in biology because of their ability to mimic nucleotides and bind to nucleotide-binding

sites, despite showing no obvious structural homology (Denzili & Piskin, 2001). Since these dyes show affinity for NAD and NADP-binding sites, we investigated their ability to be recognised by and activate the NAADP receptor in sea urchin eggs.

The binding and Ca^{2+}-release data clearly show that certain triazine dyes are capable of binding to the NAADP receptor. This represents, to our knowledge, the first report of competitive ligands for the receptor, which are unrelated structurally. Although this binding is competitive, it cannot yet be established whether NAADP and the dye bind to the same site or to two distinct binding sites that are mutually exclusive. Nonetheless, since these drugs have been shown to interact with a number of proteins that contain pyridine nucleotide-binding sites, this hints to the possibility that they might be competing for the NAADP-binding pocket (Denzili & Piskin, 2001). It is interesting to note that not all of the triazine dyes bind in an irreversible manner. Furthermore, RR120, which displays reversible characteristics, is capable of inducing Ca^{2+} release *via* the NAADP receptor. These data would seem to suggest firstly that the irreversible manner in which NAADP binds to its receptor in the sea urchin egg is a property of the ligand and not an innate property of the receptor–ligand interaction. Secondly, it suggests that the opening of the channel is neither dependent on nor determines the locking of the ligand. This is substantiated by the recent observation that, in the absence of K^+ ions, NAADP does not appear to bind in an irreversible manner, yet still releases Ca^{2+} (Dickinson & Patel, 2003).

The Ca^{2+} release induced by RR120 is mediated, at least in part, by the NAADP receptor, as shown by the effects of desensitising the receptor by pretreatment with 3 nM NAADP. Blockade of IP_3 or ryanodine receptors using heparin and ruthenium red, respectively, led to a modest increase in the Ca^{2+} release, suggesting that these mechanisms are not activated by RR120. Furthermore, RR120- and NAADP-induced Ca^{2+} release were nonadditive, suggesting that both agents release from the same Ca^{2+} pool. The NAADP-sensitive pool has been shown, in the sea urchin egg, to be distinct from those sensitive to IP_3 and cADPR (Lee & Aarhus, 1995; Genazzani & Galione, 1996b), and has been shown to reside in a lysosome-like organelle (Churchill *et al*., 2002). Nonetheless, it cannot be excluded that part of the Ca^{2+} release induced by RR120 could be due to the actions of this compound on other components of the Ca^{2+}-homeostasis machinery such as nucleotide-dependant pumps. This is substantiated by the residual Ca^{2+} release induced by RR120 when NAADP receptors are inactivated (Figure 2). Although it has been previously shown that in other systems these dyes activate the ryanodine receptor (Xu *et al*., 1989), the absence of a reduction in the Ca^{2+} release in the presence of ruthenium red suggests that the ryanodine receptors in the sea urchin egg are not activated by RR120. Furthermore, similar data obtained with heparin would also seem to exclude the possibility that RR120 can activate IP_3 receptors, as has been shown for other triazine dyes (Bootman *et al*., 1990).

The affinity shown by these dyes was utilised to test whether they could be used in an affinity chromatography protocol. Indeed, both immobilised RG5 and immobilised RG19 were able to bind a large amount of solubilised receptor and elution of the bound receptor could be induced by increasing salt concentrations. Furthermore, differences in the salt requirements for elution give rise to the possibility of combining these steps for a greater purification fold. While affinity chromatography using these dyes is unable to provide a complete purification of the receptor, it may prove to be a useful step in a purification strategy.

In conclusion, we have shown that triazine dyes, which are unrelated structurally to NAADP, are capable of binding and activating the NAADP receptor channel. Since research in the NAADP field is hindered by the lack of specific agents, it is possible that other smaller molecules might emerge from these observations with agonistic or antagonistic activities. Furthermore, the coupling of these dyes to immobilised resins does not significantly reduce their affinity for the receptor. Since the nature of this receptor has not yet been determined, it is possible that the combining of a series of purification steps, including this one, might allow the purification of the receptor protein.

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