Suramin and the suramin analogue NF307 discriminate among calmodulin-binding sites

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Calmodulin-binding sites on target proteins show considerable variation in primary sequence; hence compounds that block the access of calmodulin to these binding sites may be more selective than compounds that inactivate calmodulin. Suramin and its analogue NF307 inhibit the interaction of calmodulin with the ryanodine receptor. We have investigated whether inhibition of calmodulin binding to target proteins is a general property of these compounds. Suramin inhibited binding of [¹²⁵I]calmodulin to porcine brain membranes and to sarcoplasmic reticulum from skeletal muscle ($IC_{50} = 4.9 \pm 1.2 \mu M$ and $19.9 \pm 1.8 \mu M$, respectively) and blocked the cross-linking of [¹²⁵I]calmodulin to some, but not all, target proteins in brain membranes by [¹²⁵I]-calmodulin. Four calmodulin-binding proteins were purified [ryanodine receptor-1 (RyR1) from rabbit skeletal muscle, neuronal NO synthase (nNOS) from Sf9 cells, G-protein $\beta\gamma$ dimers ($G\beta\gamma$) from porcine brain and a glutathione S-transferase-fusion protein comprising the C-terminal calmodulin-binding

domain of the metabotropic glutamate receptor 7A (GST-CmGluR7A) from bacterial lysates]. Three of the proteins employed ($G\beta\gamma$, GST-CmGluR7A and RyR1) display a comparable affinity for calmodulin (in the range of 50–70 nM). Nevertheless, suramin and NF307 only blocked the binding of $G\beta\gamma$ and RyR1 to calmodulin–Sepharose. In contrast, the association of GST-CmGluR7A and nNOS was not impaired, whereas excess calmodulin uniformly displaced all proteins from the matrix. Thus suramin and NF307 are prototypes of a new class of calmodulin antagonists that do not interact directly with calmodulin but with calmodulin-recognition sites. In addition, these compounds discriminate among calmodulin-binding motifs.

Key words: $\beta\gamma$ dimer, G-protein, nitric oxide synthase, ryanodine receptor.

INTRODUCTION

The Ca^{2+} -binding protein calmodulin exerts a pivotal role in a host of intracellular signalling pathways. It acts as a Ca^{2+} sensor and confers the regulatory signal to target proteins (for a review, see [1]). Calmodulin contains four Ca^{2+} binding sites (helix-loop-helix, EF hands). If saturated with Ca^{2+} , calmodulin exposes a hydrophobic domain that serves as binding site for several structurally unrelated compounds; these ligands include phenothiazines (e.g. trifluoperazine, calmidazolium), peptide venoms from insects (e.g. melittin from *Apis mellifera*, mastoparan from *Vespula lewisii*) and the W series of aminoalkyl-naphthalene sulphonamides (e.g. W-7). When complexed to these ligands, calmodulin fails to regulate its targets; hence, these compounds are referred to as calmodulin antagonists (for a review, see [2]).

There are, in addition, examples of irreversible inactivation of calmodulin, e.g. by the fungal metabolite ophiobolin A [3], by the α_1 -adrenoceptor antagonist phenoxybenzamine [4,5] and by modified phenothiazines, e.g. fluphenazine-N-mustard [6]. The resulting calmodulin adducts fail to activate target enzymes and act as competitive inhibitors of unmodified calmodulin. This is, however, not true for all targets and individual ligand–calmodulin adducts differ in their capacity to activate target enzymes [6]. These findings point to the possibility that the interaction between calmodulin and individual targets may be specifically disrupted by low-molecular-mass compounds. Because there is considerable

variation in the primary sequence of calmodulin-recognition sites, it is sensible to predict that these inhibitors will be more selective than those that act on calmodulin. We have previously reported that suramin and its analogue NF307 blocked the interaction between the skeletal-muscle ryanodine receptor (ryanodine receptor-1) and calmodulin [7]. In the present work, we have investigated whether these compounds discriminated between different calmodulin-binding sites.

MATERIALS AND METHODS

Materials

The polyclonal antiserum 7 ($G\beta$ -common) [8] was raised against amino acids 8–21 of the G-protein β_1 subunit; it corresponds to the original antiserum K521 [9]. [¹²⁵I]Calmodulin (1950 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.), and dimethyl suberimidate, disuccinimidyl suberate and reagents for enhanced chemiluminescence detection were from Pierce (Rockford, IL, U.S.A.). Calmodulin–Sepharose 4B was from Amersham Pharmacia Biotech (Uppsala, Sweden). Bovine brain calmodulin was purchased from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose filters were from Sartorius (Göttingen, Germany). Buffers and salts were from Merck (Darmstadt, Germany). Suramin and mastoparan was from Calbiochem

Abbreviations used: nNOS, neuronal isoform of NO synthase; GST, glutathione S-transferase.

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(La Jolla, CA, U.S.A.), and mastoparan-X was from American Peptide Company (Sunnyvale, CA, U.S.A.). The suramin analogue NF307 [8,8'-(piperazine-1,4-diylbis(carbonylimino-4,1-phenylene)carbonylimino-4,1-phenylene)carbonylimino-bis(naphthalene-1,3,6-trisulphonic acid)], in which the central urea bridge of suramin is replaced by piperazine ring and the sulphonate groups on the naphthyl rings are in the positions 1, 3 and 6] was a generous gift of Dr P. Nickel (University of Bonn, Bonn, Germany).

Membrane preparation and protein purification

Membranes were prepared from porcine cerebral cortex as described previously [10]. Ryanodine receptor-1 was purified from heavy sarcoplasmic reticulum of rabbit skeletal muscle [7]. Purification of G-protein $\beta\gamma$ subunits from porcine brain membranes was done according to [10]. The neuronal isoform of NO synthase (nNOS) was overexpressed in Sf9 cells [11] and purified according to [12]. The creation, bacterial expression and purification of a fusion protein (GST-CmGluR7A) comprising glutathione S-transferase (GST) and the C-terminus of the metabotropic glutamate receptor 7A, mGluR7A, have been described in [13].

[125 I]Calmodulin-binding experiments

Binding of [125 I]calmodulin was done according to [14] with the following modifications. In order to remove endogenous calmodulin, porcine brain membranes or heavy sarcoplasmic reticulum from rabbit skeletal muscle were resuspended three times in 20 mM Hepes/NaOH, pH 7.5/1 mM EDTA/1 mM EGTA and subsequently sedimented by centrifugation (10 min at 20000 g). Thereafter, the membranes were used for the binding reaction; for inhibition of [125 I]calmodulin binding, porcine brain membranes (15–25 μ g) and sarcoplasmic reticulum membranes (60–90 μ g) were incubated in buffer (20 mM Hepes/NaOH, pH 7.5/1 mM EDTA/1 mM EGTA/2.1 mM CaCl_2 /100 μ M PMSF/1 μ M leupeptin) containing suramin concentrations ranging from 0.1 to 300 μ M. The reaction was started by the addition of [125 I]calmodulin (30 nM; specific radioactivity adjusted with unlabelled calmodulin to 30 c.p.m./fmol) and carried out at 25 °C for 90 min. Thereafter, bound and free ligand were separated by filtration over nitrocellulose filters (presoaked in buffer supplemented with 1 % BSA). The filters were rinsed three times in 5 ml of ice-cold buffer (20 mM Hepes/NaOH, pH 7.5/1 mM EDTA/1 mM EGTA/2.1 mM CaCl_2). Non-specific binding was determined in the presence of 10 μ M unlabelled calmodulin and amounted to less than 5 % of total binding.

Alternatively, the effect of suramin on the dissociation of [125 I]calmodulin was investigated as follows; porcine brain membranes (15–25 μ g) and heavy sarcoplasmic reticulum (60–90 μ g) membranes were incubated with 30 nM [125 I]calmodulin for 90 min. Dissociation was initiated by adding 1 μ M calmodulin, 100 μ M suramin or the combination of calmodulin and suramin. The dissociation reactions were terminated at intervals between 30 s and 60 min by filtration over nitrocellulose filters.

Cross-linking experiments

Porcine membranes, pretreated as described above (20 μ g/reaction) were diluted in buffer (20 mM Hepes/NaOH, pH 7.5/1 mM EDTA/1 mM EGTA/2.1 mM CaCl_2). Subsequently NF307, suramin and calmodulin (concentrations indicated in the Figure legends) were added. The reaction was initiated by the

addition of [125 I]calmodulin (35 nM) in a final volume of 25 μ l and allowed to proceed for 30 min at 25 °C. Thereafter, the membranes were sedimented by centrifugation (10 min at 20000 g), the supernatant was aspirated to remove unbound [125 I]calmodulin and buffer containing dimethyl suberimidate was added to give a final concentration of 100 μ M. The membranes were resuspended by sonication and the samples were incubated at 20 °C for 5 min; the reaction was quenched by adding Tris/HCl (pH 7.5; final concentration 50 mM). Alternatively, the centrifugation step was omitted and the cross-linker was added directly to the incubation mixture. There was no appreciable difference between the two approaches. The samples were mixed with Laemmli buffer (supplemented with 40 mM dithiothreitol as the reducing agent), heated at 40 °C for 30 min and subjected to SDS/PAGE. The proteins were stained with Coomassie Brilliant Blue and the gel was dried for autoradiography (Kodak X-Omat, exposure for 0.5–7 days). For cross-linking of mastoparan and mastoparan-X with calmodulin, 2 μ g of calmodulin and 0.2 μ g of mastoparan or mastoparan-X were diluted in buffer (20 mM Hepes/NaOH, pH 8/100 μ M CaCl_2) in the absence or presence of suramin and NF307 (at concentrations ranging from 10–300 μ M) to give a volume of 30 μ l. After incubating at 30 °C for 30 min, disuccinimidyl suberate was added to a final concentration of 1 mM. The samples were incubated for 30 min at 30 °C. Finally, the samples were mixed with Laemmli buffer, heated at 90 °C for 5 min, applied to SDS/PAGE and stained with Coomassie Brilliant Blue.

Binding to calmodulin–Sephacrose

The purified proteins (1–5 μ g of ryanodine receptor-1, 0.5–5 μ g of G-protein $\beta\gamma$ subunits, 3–10 μ g of GST-CmGluR7A and 2–5 μ g of nNOS) were diluted in reaction buffer (100 μ l final volume), the composition of which was optimized for each protein to give maximum binding to the calmodulin–Sephacrose. For the ryanodine receptor-1, the buffer was 200 mM KCl, 10 mM NaCl, 20 mM Hepes/NaOH, pH 7.4, 8 mM Chaps, 0.68 % phosphatidylcholine, 1 mM EGTA and 1.2 mM CaCl_2 ; for G-protein $\beta\gamma$ dimers it was 200 mM KCl, 10 mM NaCl, 20 mM Hepes/NaOH, pH 7.4, 1 mM EGTA, 2 mM CaCl_2 and 8 mM Chaps; for nNOS it was 150 mM NaCl, 50 mM Tris/HCl, pH 7.0, 1 mM EGTA, 1.2 mM CaCl_2 , 0.1 % β -mercaptoethanol and 20 % (v/v) glycerol; and for the GST-CmGluR7A the buffer was 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 8 mM Chaps, 1 mM EDTA and 2 mM CaCl_2 .

Two approaches were employed, namely (i) inhibition of binding and (ii) elution. For inhibition of binding the proteins were first incubated in the absence or presence of calmodulin (10 μ M), NF307 (100 μ M) or suramin (300 μ M). In some instances, CaCl_2 was omitted and the incubation was carried out in the sole presence of EGTA to evaluate the extent of binding in a nominally Ca^{2+} -free solution. Thereafter, calmodulin–Sephacrose (40 μ l of a 50 % slurry pre-equilibrated in the appropriate buffer) was added. After an incubation period of 60 min at 4 °C, the suspension was centrifuged for 5 min at 500 g. The supernatant was withdrawn and the matrix was resuspended in the appropriate reaction buffer (100 μ l). This washing step was repeated three times. Alternatively, the proteins were first allowed to bind to the calmodulin–Sephacrose; subsequently, calmodulin, suramin, NF307 or EGTA were added and their ability to release the bound proteins from the matrix was assessed (elution protocol). The supernatant containing the non-adsorbed proteins as well as the supernatant of the individual washing steps were mixed with Laemmli sample buffer and subjected to heat

denaturation (5 min at 95 °C). Similarly, the proteins that remained adsorbed to the calmodulin–Sephacrose after the last wash were released by boiling after the addition of Laemmli buffer. Aliquots of each fraction (corresponding to 20–50 % of the total volume) were applied to SDS/polyacrylamide gels. The proteins were visualized by staining with Coomassie Brilliant Blue, silver or by immunoblotting, as indicated in the legends to Figures 3 and 4.

RESULTS AND DISCUSSION

Inhibition of [125 I]calmodulin binding to porcine brain membranes and sarcoplasmic reticulum from rabbit skeletal muscle

Suramin inhibits the action of calmodulin on the ryanodine receptor [7] and suppresses binding of [125 I]calmodulin to sarcoplasmic reticulum [15]. In order to test if the apparent competition between suramin and calmodulin is restricted to the ryanodine receptor, we have compared the effects of suramin on binding of [125 I]calmodulin to heavy sarcoplasmic reticulum and to porcine brain membranes. The heavy fraction of the sarcoplasmic reticulum is highly enriched with the ryanodine receptor, which represents the predominant calmodulin-binding protein. In contrast, cerebrocortical membranes contain many different calmodulin-binding proteins. As can be seen from Figure 1(A), binding of [125 I]calmodulin to cerebrocortical membranes is more sensitive to inhibition by suramin ($IC_{50} = 4.9 \pm 1.2 \mu M$; $n = 3$) than the binding to sarcoplasmic reticulum ($IC_{50} = 19.9 \pm 1.8 \mu M$; $n = 3$). Thus the observations show that the action of suramin is not confined to the ryanodine receptor; a potent

action of suramin can also be observed in cerebrocortical membranes, a preparation that contains a large variety of targets for calmodulin.

Cross-linking of [125 I]calmodulin to porcine brain membranes

The slopes of the inhibition curves shown in Figure 1(A) differ. In sarcoplasmic reticulum, the inhibition curve was monophasic with a Hill coefficient that was not different from 1 ($h = 1.01 \pm 0.03$). In contrast, in porcine brain membranes, the displacement curve was shallow ($h = 0.64 \pm 0.08$). A Hill coefficient of < 1 suggested the presence of calmodulin-binding sites that varied in their affinity for suramin. We have therefore visualized the interaction of [125 I]calmodulin with its target proteins in porcine brain membranes by carrying out cross-linking reactions in the absence and presence of suramin. Obviously, this approach preferentially identifies abundant target proteins; it is limited by the fact that cross-linked multimeric proteins may be poorly resolved and that the efficiency of cross-linking with sub-erimidates varies with the availability of free amino groups.

As expected, [125 I]calmodulin was incorporated into many membrane proteins, several of which were reasonably well resolved by electrophoresis in the range of 95–40 kDa; this is best seen in the short exposure (Figure 1B, left-hand control lane); in these short exposures, labelling is barely detectable in the presence of excess unlabelled calmodulin (results not shown). We therefore increased the time of autoradiography. This resulted in overexposure of the control lane (Figure 1B, right-hand control lane), but allowed us to compare the formation of cross-

Figure 1 (A) Inhibition of [125 I]calmodulin binding to porcine brain membranes and sarcoplasmic reticulum membranes by suramin, and (B) cross-linking of [125 I]calmodulin to porcine brain membranes

(A) Porcine brain membranes (15–25 μg , $\circ$) and sarcoplasmic reticulum (60–90 μg , HSR, $\bullet$) were incubated in the presence of 30 nM [125 I]calmodulin and of the indicated concentrations of suramin under conditions outlined in the Materials and methods section. Specific binding observed in the absence of suramin was set at 100% and amounted to 0.8–1.6 pmol/mg (sarcoplasmic reticulum) and 7.5–12.5 pmol/mg (porcine brain membranes). Data are means $\pm$ S.D. from three independent experiments (with different preparations of both radioligand and membranes) carried out in duplicate. (B) Porcine brain membranes (20 μg) were incubated with [125 I]calmodulin in the absence (control) or presence of 300 μM suramin (SUR), 100 μM NF307 or 1 μM calmodulin (CaM) as outlined in the Materials and methods section. Cross-linking was initiated by the addition of 100 μM dimethyl suberimidate. Aliquots of the samples (50%) were applied on to a denaturing gel. The left-most lane (control-s) represents a short (overnight) exposure of the adjacent control lane to illustrate that the bands at 97, 70 and 60 kDa can also be detected as distinct entities under control conditions. The histogram shows the means $\pm$ S.E.M. from three densitometric quantifications using shorter exposures; the intensities recorded for the bands migrating at $\approx$ 97, 70 and 60 kDa in the control lane were set to 1.0 and used to normalize the intensities determined in the presence of suramin, NF307 and calmodulin.

Figure 2 Inhibition by suramin and NF307 of the formation of the ≈ 97 kDa (top panel) and ≈ 60 kDa (bottom panel) cross-linking products

Assay conditions were similar to those used in Figure 1(B). Brain membranes were incubated in the absence (control, Ctl) and presence of the indicated concentrations of suramin (SUR), NF307 and calmodulin (CaM); no XL in the top panel refers to an incubation performed without cross-linker. The histograms show means $\pm$ S.E.M. ($n = 3$); the intensity recorded by densitometry in the control lane was set to 1.0 and used to normalize the intensities determined in the presence of suramin, NF307 and calmodulin.

linked products in the presence of suramin, NF307 and calmodulin. Suramin and the suramin analogue NF307 suppressed the incorporation of [125 I]calmodulin (Figure 1B). However, the extent to which this inhibition was seen at the level of individual proteins differed greatly. For instance, labelling of the band migrating at ≈ 97 kDa was essentially abolished in the presence of suramin or NF307, whereas the incorporation of [125 I]-calmodulin into the band migrating at ≈ 70 kDa was reduced by suramin and enhanced by NF307. In contrast, addition of unlabelled calmodulin (at 1 μ M) uniformly reduced formation of all radiolabelled species (Figure 1B). Addition of 10 μ M calmodulin suppressed the formation of radioactively labelled cross-linking products to barely detectable levels (results not shown; see also Figure 2). Previously, NF307 was found to be more potent than suramin in blocking the action of Ca^{2+} -liganded calmodulin on the ryanodine receptor type-1 [7].

We have used the formation of the ≈ 97 kDa and ≈ 60 kDa cross-linking products as indicators to determine the concentration range in which suramin and NF307 blocked the incorporation of [125 I]calmodulin. It is evident from Figure 2 (top panel) that NF307 was substantially more potent than suramin in inhibiting the formation of the ≈ 97 kDa radiolabelled species. This, apparently, does not pertain to all calmodulin-binding sites; suramin and NF307 did not differ in their abilities to suppress formation of the ≈ 60 kDa cross-linking product (Figure 2, bottom panel) and, as mentioned above, addition of NF307 actually favoured the appearance of the ≈ 70 kDa cross-linking product (Figure 1B). We are at loss to explain this latter finding. Nevertheless, the current observations are consistent with the hypothesis that modest variations in the structure of suramin affect the affinity of the compounds for binding to calmodulin-recognition sites.

Figure 3 Immobilization on calmodulin-Sepharose of the ryanodine receptor-1 (A), G-protein $\beta\gamma$ dimers (B), purified nNOS (C) and GST-CmGluR7A (D)

The proteins were incubated with pre-equilibrated calmodulin-Sepharose in binding buffer, as described in the Material and methods section, in the absence (control) and presence of 10 μ M calmodulin (CaM), 300 μ M suramin (SUR), 100 μ M NF307, or (C only) 1 mM EGTA. The individual samples were centrifuged and the matrix was washed three times with binding buffer; the supernatants from the initial incubation (s) and the last wash (w) were saved. The residual matrix-bound protein was released by boiling in Laemmli sample buffer (m). The experiments depicted in (A) and (C) were carried out in parallel with comparable amounts of protein in the input (i; 5 μ g/assay). Aliquots corresponding to one-fifth of each fraction (i, s, w, m) were applied to SDS/polyacrylamide gels and the proteins were detected by silver staining. In (B) and (D), aliquots corresponding to one-third of the input (i), and one-half of the first supernatant (s), the last wash (w) and the residual matrix-bound protein (m) were applied to denaturing gels and the proteins were visualized by immunodetection with the anti-G β /common-specific antiserum 7 (B) or by staining with Coomassie Brilliant Blue (D).

Calmodulin affinity chromatography

Taken together, the data summarized in Figures 1 and 2 provide circumstantial evidence for selective recognition of calmodulin-binding motifs by suramin and NF307. In order to confirm this interpretation, we employed four purified proteins that are known to bind calmodulin. These were the ryanodine receptor-1 (Figure 3A), G-protein $\beta\gamma$ dimers (Figure 3B), nNOS (Figure 3C) and GST-CmGluR7A (Figure 3D). Purified ryanodine receptor-1 was visualized as a doublet with bands migrating in the range of 300–350 kDa (Figure 3A). Partial proteolysis is virtually always

Figure 4 Difference in the elution of G-protein $\beta\gamma$ dimers (A) and GST-CmGluR7A (B) from calmodulin-Sepharose by suramin and NF307

G-protein $\beta\gamma$ dimers (A) and GST-CmGluR7A (B) were incubated for 1 h at 4 °C in binding buffer; after three washes in binding buffer, stepwise elution (e1–e3) was done with binding buffer containing 10 μ M calmodulin (CaM), 100 μ M NF307 or 300 μ M suramin (SUR). Aliquots corresponding to 30% of the input (i), 50% of the three successive elution steps (e1–e3) and to 30% of the protein bound to the matrix (m) released by denaturation of calmodulin-Sepharose were applied to SDS/polyacrylamide gels. Proteins were visualized by immunodetection with the G β common-specific antiserum 7 (A) or by staining with Coomassie Brilliant Blue (B).

observed during purification of the ryanodine receptor-1. In immunoblots, both bands reacted with a monoclonal antibody directed against the ryanodine receptor-1 (results not shown, see also [7]). In the absence of any inhibitor, essentially all of the ryanodine receptor-1 was bound to calmodulin immobilized on the Sepharose matrix (Figure 3A, see control and input lanes). If the ryanodine receptor-1 was incubated with calmodulin, suramin or NF307, association with the matrix was suppressed and the protein was recovered in the supernatant (Figure 3A). Analogous observations were made with the G-protein $\beta\gamma$ dimers (Figure 3B).

In contrast, suramin and NF307 did not inhibit binding of nNOS (Figure 3C) and GST-CmGluR7A (Figure 3D) to calmodulin-Sepharose; indeed, essentially all of the protein was recovered in the matrix. We note that in Figure 3(C) a modest amount of nNOS was recovered in the matrix in the presence of calmodulin (Figures 3B–3D, compare lanes m under CaM). This may reflect non-specific adsorption of the protein to the Sepharose because it was also observed in a nominally Ca^{2+} -free solution in the presence of EGTA (Figure 3C). More importantly, this material represents only a minor fraction of the total protein ($\leq 10\%$ of the input). Hence, we do not believe that it confounds the interpretation. We conclude that the calmodulin-binding sites of the ryanodine receptor-1 and of G-protein $\beta\gamma$ dimers are sensitive to blockage by suramin analogues whereas those of nNOS and of the C-terminus of mGluR7A are not.

We stress that analogous results were obtained if suramin and NF307 were added to release the proteins prebound to the matrix, i.e. suramin and NF307 eluted the ryanodine receptor and G-protein $\beta\gamma$ dimers but not nNOS and GST-CmGluR7A from the calmodulin-Sepharose (shown for G-protein $\beta\gamma$ dimers and GST-CmGluR7A in Figures 4A and 4B, respectively). In contrast, incubation with calmodulin uniformly reduced the amount of protein that was recovered in the matrix (Figure 4).

Calmodulin-binding motifs differ widely with respect to their affinity for calmodulin. There are, in particular, several proteins that contain calmodulin as a permanently bound regulatory subunit, while others only bind Ca^{2+} -liganded calmodulin with appreciable affinities (typically in the range of 5–100 nM). Accordingly, calmodulin-binding motifs vary in their primary sequence; the conserved features comprise α -helical stretches enriched in hydrophobic amino acids and clusters of basic amino acids [16]. The affinity of nNOS for Ca^{2+} -liganded calmodulin is about 5 nM [17]. In contrast, the three other proteins employed bind Ca^{2+} -liganded calmodulin with comparable affinities, i.e. 45 nM for the ryanodine receptor [7], ≈ 40 nM for $\beta\gamma$ dimers [18] and 57 nM for the mGluR7 C-terminal tail [13]. Thus the ability of suramin and NF307 to discriminate between calmodulin-binding sites cannot result solely from differences in the affinity of these sites for Ca^{2+} -liganded calmodulin.

Suramin does not interact with core calmodulin-binding motifs and inhibits calmodulin binding allosterically

The wasp venoms mastoparan and mastoparan-X represent short peptides of 14 amino acids that bind to calmodulin with high affinity via different recognition motifs (1,8,14-motifs types A and B [16]). Because of their small sizes, mastoparan and mastoparan-X can be viewed as minimum calmodulin-binding motifs (of about four helical turns). We tested whether suramin and NF307 interfered with the interaction of mastoparan or mastoparan-X with calmodulin. At concentrations where the compounds effectively suppressed the interaction with calmodulin-binding proteins, neither suramin nor NF307 prevented the cross-linking of mastoparan or mastoparan-X (results not shown) to calmodulin. It is evident that the core calmodulin-binding motif does not predict whether suramin and NF307 block binding of calmodulin; the calmodulin-binding sites in the ryanodine receptor-1, nNOS and G-protein β subunit all conform to a type A 1,8,14-motif, whereas the C-terminus of mGluR7A contains a type B 1,8,14-motif [16].

Taken together, these observations indicate that the additional residues in the sequences that flank the core calmodulin-binding domains rather than the core domains themselves must be important in determining the affinity of suramin and NF307. If this interpretation is correct, suramin ought to inhibit the binding of [125 I]calmodulin in an allosteric manner. This was tested by comparing the ability of suramin and of unlabelled calmodulin to dissociate [125 I]calmodulin prebound to target proteins. In both porcine brain membranes (Figure 5A) and sarcoplasmic reticulum (Figure 5B) addition of 1 μ M unlabelled calmodulin resulted in the slow release of [125 I]calmodulin (Figure 5, ●). The resulting curve was described adequately by the equation for a mono-exponential decay (with rate constants, k , of $0.007 \pm 0.001 \text{ min}^{-1}$ and $0.011 \pm 0.002 \text{ min}^{-1}$ in porcine brain membranes and sarcoplasmic reticulum, respectively). If the concentration of unlabelled calmodulin was increased to 10 μ M, the rate was not affected (results not shown).

In contrast, addition of suramin resulted in the very rapid dissociation of about 30% of the bound radiolabelled calmodulin whereas the remaining calmodulin was slowly released (Figure 5, ▼). Accordingly, the reaction was best described by the sum of two exponential decay curves (with rate constants of $k_1 = 0.56 \pm 0.18$ and $1.97 \pm 1.43 \text{ min}^{-1}$ and $k_2 = 0.006 \pm 0.001$ and $0.027 \pm 0.004 \text{ min}^{-1}$ in porcine brain membranes and sarcoplasmic reticulum, respectively). If suramin and calmodulin were added in combination (Figure 5, ■), there was no appreciable difference to the dissociation observed in the sole presence of suramin ($k_1 = 0.38 \pm 0.16$ and $3.47 \pm 2.24 \text{ min}^{-1}$ and $k_2 =$

Figure 5 Dissociation of [125 I]calmodulin from porcine brain membranes (A) and sarcoplasmic reticulum (B)

Porcine brain membranes (15–25 μ g; A) and sarcoplasmic reticulum (60–90 μ g; B) were preincubated with 30 nM [125 I]calmodulin as outlined in the Materials and methods section. After 90 min ($t = 0$ on the y axis), an aliquot was withdrawn to determine the amount of prebound radioligand (○) and dissociation was initiated by adding 1 μ M calmodulin (●), 100 μ M suramin (▼) or both (■). At the indicated time points, the dissociation of [125 I]calmodulin was terminated by filtration over nitrocellulose filters. Under control conditions [125 I]calmodulin binding remained stable for up to 60 min (○). The level of prebound [125 I]calmodulin was set to 100% to normalize for inter-assay variation and amounted to 4.6–12.4 pmol/mg (A) and 0.9–1.4 pmol/mg (B). Data are means $\pm$ S.D. from three independent experiments carried out in duplicate.

0.008 $\pm$ 0.002 and 0.022 $\pm$ 0.003 min $^{-1}$ in porcine brain membranes and sarcoplasmic reticulum, respectively). Thus the kinetics of dissociation in the presence of suramin differed from those obtained by displacing [125 I]calmodulin with unlabelled calmodulin. This can only be accounted for by an allosteric mechanism of action where suramin has access to residues that

are not occupied by calmodulin but which regulate the affinity of the calmodulin-binding site. Finally, the difference between the monophasic and biphasic dissociation kinetics observed in the presence of calmodulin and suramin, respectively, also shows that suramin discriminates among calmodulin-binding sites in a manner that is independent of their affinity for calmodulin.

Because signalling via Ca $^{2+}$ -liganded calmodulin controls an array of cellular functions ranging from proliferation to smooth-muscle-cell contraction, in the past calmodulin and calmodulin-recognition motifs have been proposed as potential drug targets [19]. Progress in this area has, however, been hampered by the lack of selectivity resulting from the non-selective inhibition that occurs if calmodulin itself is targeted by the currently available calmodulin antagonists. The drawback of suramin is its propensity to interact with many mammalian enzymes, receptors and additional signalling proteins (reviewed in [20], see also [21]). However, in the instances where the structure–activity relationship has been explored with individual targets, large differences in the potency of suramin analogues have been observed [22,23] and very modest structural modifications of NF307 lead to a complete loss in the ability to activate the ryanodine receptor-1 via calmodulin-binding sites [7]. It is therefore conceivable that the suramin analogue NF307 could serve as a lead compound for a novel class of calmodulin inhibitors that target distinct special subsets of calmodulin-recognition motifs.

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