Cyclosporin A augments angiotensin II-stimulated rise in intracellular free calcium in vascular smooth muscle cells

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Pretreatment of rat vascular smooth muscle cells with the immunosuppressive drug cyclosporin A caused concentration- and time-dependent increases in both the amplitude and duration of the angiotensin II-induced rise in cytosolic free calcium, as measured with quin 2. Cyclosporin A had no significant effect on basal quin 2 fluorescence. However, cyclosporin A increased the basal $^{45}\text{Ca}^{2+}$ influx. This stimulation of $^{45}\text{Ca}^{2+}$ influx was not blocked by nifedipine (10^{-6} M). Cyclosporin A also augmented the angiotensin II-stimulated influx and efflux of $^{45}\text{Ca}^{2+}$. These results demonstrate that cyclosporin A increases the permeability of the plasma membrane for Ca^{2+} and also augments the angiotensin II-induced increases in cytosolic free calcium.

INTRODUCTION

Cyclosporin A (CsA), a fungal cyclic polypeptide, is an immunosuppressive drug that acts primarily on T-lymphocytes [1]. This immunosuppression seems to be mediated by inhibition of the production of T-lymphocyte-derived lymphokines [2,3]. The use of cyclosporin A as a powerful tool in the management of graft rejection is hampered by side effects, one of them being the development of arterial hypertension in some of the patients [4]. It has been suggested that hypertension results from an enhanced contraction of vascular smooth muscle cells (SMC) induced by CsA [5,6]. Smooth muscle contraction is initiated by a receptor-dependent increase of $[\text{Ca}^{2+}]_i$ (see [7] for review), which is brought about by the release of calcium from intracellular stores and by an increased influx of calcium from the extracellular space. Since it has been reported that CsA alters the cellular calcium homeostasis in hepatocytes [8] and T-lymphocytes [9], we studied the effects of CsA on the angiotensin II-induced $[\text{Ca}^{2+}]_i$ transients in vascular SMC prepared from rat aorta.

The present report demonstrates that CsA treatment increases the permeability of the plasma membrane of SMC to calcium and increases the $[\text{Ca}^{2+}]_i$ response to stimulation with the vasoconstrictive hormone angiotensin II. This may account for the hypertensive side-effects of CsA treatment.

EXPERIMENTAL

Cell culture

Cultures of SMC from rat aorta were prepared by the explant technique described by Ross [10]. Aortas were taken from male Sprague–Dawley rats (200–300 g body weight). The vessels were thoroughly freed from adventitial tissue and cut into pieces of 1–2 mm in size. These were seeded in 25 cm² culture flasks (Primaria, Falcon, Basel, Switzerland) with 5 ml of medium (Dulbecco's

modified Eagle's medium) supplemented with 15 % fetal calf serum, glutamine, and antibiotics, and were incubated in a humidified atmosphere in air/CO₂ (19:1). Medium was removed after 7 days, and tissue explants were detached using a solution of Hanks' balanced salt solution (Ca^{2+} and Mg^{2+} free) supplemented with 2 mM-EDTA and 0.25 % trypsin (Sigma). Single cells were separated from tissue pieces by sedimentation at 1 g and were cloned by limited dilution using 96-microwell plates. Clones with apparent SMC morphology were used for further processing. Demonstration of the intermediate filament desmin, which is considered to be specific for myogenic cells [11], was done according to Osborn & Weber [12,13]. Clonal cells were grown in Falcon culture dishes and flasks and were passaged twice a week at a 1:3 ratio. Cells were fed every other day with medium as above. For experiments, confluent cultures between passages 5 and 15 were used.

Measurement of $^{45}\text{Ca}^{2+}$ influx and $[\text{Ca}^{2+}]_i$

Influx of $^{45}\text{Ca}^{2+}$ was measured 15, 45, 75 and 105 s after the addition of 1 ml of HBS, consisting of 20 mM-Hepes, 148 mM-NaCl, 5 mM-KCl, 2 mM-CaCl₂, 1 mM-MgCl₂ and 6 mM-glucose, pH 7.35, supplemented with 4 μCi of $^{45}\text{Ca}^{2+}$ to cells in 35 mm diameter dishes. Calculations were done as described previously [14].

$[\text{Ca}^{2+}]_i$ was measured using the fluorescent calcium indicator quin 2 as described [15]. In brief, approx. 2×10^7 cells were incubated with 25 μM -quin 2/AM in Dulbecco's modified Eagle's medium for 30 min followed by another 30 min incubation with 4 vol. of the medium. Aliquots of 10^6 cells were washed twice and resuspended in HBS containing 1.3 mM- Ca^{2+} . Fluorescence of quin 2-loaded cells was measured at 37 °C in a Shimadzu RF-510 spectrofluorophotometer using an excitation wavelength of 340 nm and an emission wavelength of 492 nm. The fluorescence signal was calibrated at the end of each individual trace as described by Tsien *et al.* [16].

Abbreviations used: CsA, cyclosporin A; $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; SMC, smooth muscle cells; HBS, Hepes-buffered saline.

Assay of $^{45}\text{Ca}^{2+}$ efflux

Cells cultured in 16 mm diameter wells were washed and incubated for 24 h at 37 °C in Dulbecco's modified Eagle's medium containing 15% fetal calf serum plus 4 μCi of $^{45}\text{Ca}^{2+}$ /ml to ensure complete labelling of exchangeable pools of Ca^{2+} . Aliquots of CsA solution or vehicle were then added and the cells were incubated for 3 h. Efflux experiments were initiated by rapidly washing the cells four times with HBS at 37 °C to remove extracellular $^{45}\text{Ca}^{2+}$ and by adding 0.5 ml of HBS. Efflux was measured for 3 $\times$ 5 min and was found to be linear after this period. Angiotensin II (10^{-7} M) or vehicle was added and efflux was measured for further 3 $\times$ 5 min. The incubation was terminated by washing the cells in ice-cold HBS followed by addition of 0.1% SDS. An aliquot was counted to determine the $^{45}\text{Ca}^{2+}$ content remaining in the cells. The remainder was used for protein determination [17] using bovine serum albumin (Sigma) as standard. The average rate of loss of $^{45}\text{Ca}^{2+}$ (expressed as fractional loss/5 min) during a given sampling interval was calculated: fractional loss = (cellular $^{45}\text{Ca}_t - \text{cellular } ^{45}\text{Ca}_{t_1}) / (\text{cellular } ^{45}\text{Ca}_{t_1} \times (t_2 - t_1))$.

Chemicals

Angiotensin II, [Sar¹,Ala⁸]angiotensin II and nifedipine were from Sigma, cyclosporin A from Sandoz, $^{45}\text{CaCl}_2$ from Amersham International, quin 2/AM from Calbiochem, cell culture media and nutrients were from Boehringer-Mannheim, and all other chemicals were from Merck.

RESULTS

Effects of CsA on angiotensin II-evoked increases in $[\text{Ca}^{2+}]_i$

Angiotensin II (100 nM) induced a rapid and pronounced increase in $[\text{Ca}^{2+}]_i$ in smooth muscle cells from rat aorta as measured with quin 2 (Fig. 1a). The $[\text{Ca}^{2+}]_i$ in unstimulated cells was calculated to be 130 ± 12 nM (mean $\pm$ S.E.M., $n = 21$). On exposure to angiotensin II, a characteristic temporal pattern of immediate and rapid increase in quin 2 fluorescence was observed. These increases reached peak values within 15–30 s and were followed by a rapid decay in fluorescence. Pretreatment of the cells with CsA had no effect on basal $[\text{Ca}^{2+}]_i$, which was calculated to be 133 ± 3 nM (mean $\pm$ S.E.M., $n = 41$). However, CsA dose- and time-dependently increased the peak value of angiotensin II-stimulated increase in $[\text{Ca}^{2+}]_i$ and delayed the decay in $[\text{Ca}^{2+}]_i$ observed thereafter (Figs. 1 and 2). This stimulatory effect of CsA was maximal at a concentration of 10 $\mu\text{g}/\text{ml}$ (8.3 μM) and half-maximal effects were observed at 2 $\mu\text{g}/\text{ml}$ (Fig. 2a). The ability of CsA to enhance the angiotensin II-induced rise in $[\text{Ca}^{2+}]_i$ was also dependent on the time of preincubation of the cells with CsA. As shown in Fig. 2(b), the stimulatory effect of CsA reached a maximal value after about 1 h of preincubation time with a half-maximal effect after 20 min. In order to establish whether the angiotensin II-induced rise in $[\text{Ca}^{2+}]_i$ requires the presence of extracellular Ca^{2+} , cell suspensions were treated with 1.3 mM-EGTA to chelate Ca^{2+} prior to stimulation. Under these conditions, 100 nM-angiotensin II elicited a rise in quin 2 fluorescence that was only slightly reduced in its peak value. However, the signal returned to basal values more rapidly than in the

Fig. 1. Effects of CsA treatment on the angiotensin II-stimulated rise in $[\text{Ca}^{2+}]_i$

Quin 2 fluorescence in response to 100 nM-angiotensin II was registered. Angiotensin II was added at time point 0. (a)–(c) show individual fluorescence traces: (a) control cells, (b) cells pretreated with CsA (1 $\mu\text{g}/\text{ml}$) for 3 h, (c) cells pretreated with CsA (10 $\mu\text{g}/\text{ml}$) for 3 h. (d) shows mean values of $[\text{Ca}^{2+}]_i$ from 5–11 experiments $\pm$ S.E.M.: ●, control cells; ○, cells pretreated with CsA (1 $\mu\text{g}/\text{ml}$) for 3 h; ▲, cells pretreated with CsA (10 $\mu\text{g}/\text{ml}$) for 3 h.

Fig. 2. Dose- and time-dependency of CsA effects on angiotensin II-induced rises in $[\text{Ca}^{2+}]_i$

Cells were loaded with quin 2 and stimulated with angiotensin II (100 nM). Peak values of $[\text{Ca}^{2+}]_i$ were measured. Data are the means $\pm$ S.E.M. for 4–11 experiments. (a) Cells pretreated for 3 h with the indicated concentrations of CsA; (b) cells preincubated with 10 $\mu\text{g}/\text{ml}$ of CsA for the indicated time period.

presence of normal extracellular calcium concentrations (Fig. 3b). This was also the case for cells pretreated with CsA, in which the peak value of angiotensin II-mobilized $[\text{Ca}^{2+}]_i$ was only slightly reduced; however, the sustained response was clearly attenuated (compare Fig. 3e with Fig. 3f). In cells pretreated with CsA the $[\text{Ca}^{2+}]_i$ was significantly higher than in control cells (Figs. 3b and 3f). This indicates that CsA enhanced the angiotensin II-induced mobilization of Ca^{2+} from intracellular stores.

When extracellular calcium was added 3 min after the addition of angiotensin II, there was a small increase in

[Ca²⁺]_i in control cells (Fig. 3b); however, a large increase in [Ca²⁺]_i in cells pretreated with CsA was observed (Fig. 3f). This second rise in [Ca²⁺]_i required the presence of angiotensin II and was prevented by the specific antagonist [Sar¹,Ala⁸]angiotensin II (Figs. 3c and 3g). However, [Sar¹,Ala⁸]angiotensin II did not inhibit the rise in [Ca²⁺]_i induced by [Arg⁸]vasopressin (100 nM) (Figs. 3c and 3g). This shows that CsA affects Ca²⁺ influx in SMC. Ca²⁺ influx appears to be involved mostly in sustaining the action of angiotensin II rather than in initiating the cellular response to the hormone.

To characterize further the angiotensin II-induced Ca²⁺ influx, we tested the effect of nifedipine. Nifedipine belongs to the dihydropyridine group of Ca²⁺ channel blockers and specifically blocks voltage-dependent Ca²⁺ channels of the L-type [18]. Pretreatment of SMC with

1 μ M-nifedipine had no influence on angiotensin II-induced rises in [Ca²⁺]_i either in control cells or in cells treated with CsA (Figs. 3d and 3h). This indicates that the angiotensin II-evoked Ca²⁺ influx occurs via Ca²⁺ channels that are not of the L-type.

Effects of CsA on angiotensin II-evoked transmembrane ⁴⁵Ca²⁺ fluxes

In order further to distinguish between possible CsA effects on angiotensin II-induced Ca²⁺ influx and intracellular Ca²⁺ mobilization, we performed tracer flux studies. It should be noted that the measurement of Ca²⁺ influx is made in non-steady state conditions. Hence what is being measured are altered Ca²⁺ fluxes across the plasma membrane, rather than enhanced net Ca²⁺ uptake. The basal ⁴⁵Ca²⁺ influx, as calculated from the slope of the regression lines, was 5762 $\pm$ 373 c.p.m./min per mg of protein (1.4 nmol of Ca²⁺/min per mg of protein) (mean $\pm$ S.E.M., *n* = 5). Angiotensin II (100 nM) increased this initial rate of influx by 110% (Table 1). CsA pretreatment increased basal ⁴⁵Ca²⁺ influx by 38% and angiotensin II-stimulated ⁴⁵Ca²⁺ influx by up to 162% of the control value. This effect of CsA was not blocked by nifedipine (1 μ M), again showing that CsA stimulates Ca²⁺ influx via non-dihydropyridine-sensitive channels. The fact that CsA increases basal ⁴⁵Ca²⁺ influx without altering [Ca²⁺]_i, as measured with quin 2, might be explained by the buffering capacity of quin 2 which makes it unable to resolve small increases in [Ca²⁺]_i.

The effect of angiotensin II on ⁴⁵Ca²⁺ efflux is shown in Table 2. In the absence of angiotensin II, a nearly constant amount of ⁴⁵Ca²⁺ was released from the cells in each 5 min period. Addition of angiotensin II increased the amount of ⁴⁵Ca²⁺ appearing in the external medium by about 3-fold. Pretreatment of SMC with CsA had no effect on the basal release of ⁴⁵Ca²⁺ but augmented the angiotensin II-stimulated ⁴⁵Ca²⁺ release by an additional 33%. As ⁴⁵Ca²⁺ efflux is a measure of the hormone-induced mobilization of intracellular calcium, these results also argue that CsA treatment augments the amount of Ca²⁺ released from intracellular stores upon activation of SMC with angiotensin II. Furthermore, these results exclude the possibility that the increased amplitude and duration of the angiotensin II-induced rise in [Ca²⁺]_i is due to an inhibition of Ca²⁺ extrusion from the cells.

Fig. 3. Effects of CsA on the angiotensin II-induced rise in [Ca²⁺]_i; dependence on extracellular calcium

Quin 2 fluorescence in response to 100 nM-angiotensin II was registered. Traces (a)–(d), control cells; traces (e)–(h), cells pretreated with CsA (10 μ g/ml) for 3 h. (a, e), angiotensin II (AII)-evoked rise in [Ca²⁺]_i; (b, f), sequential addition of EGTA (1.3 mM), angiotensin II (100 nM) and CaCl₂ (5 mM); (c, g), sequential addition of [Sar¹,Ala⁸]angiotensin II (Sar) (1 μ M), EGTA (1.3 mM), angiotensin II (100 nM), CaCl₂ (5 mM) and [Arg⁸]vasopressin (AVP) (100 nM); (d, h), sequential addition of nifedipine (Nif) (1 μ M) and angiotensin II (100 nM).

Table 1. Effect of CsA on basal and angiotensin II-stimulated ⁴⁵Ca²⁺ uptake in smooth muscle cells

The cells were pretreated with CsA (10 μ g/ml) for 3 h and ⁴⁵Ca²⁺ influx was measured as outlined under 'Experimental'. Results are means $\pm$ S.E.M. for *n* = 5. **P* < 0.01 versus control, ***P* < 0.05 versus angiotensin II, by Student's *t*-test.

Addition	⁴⁵ Ca ²⁺ influx (c.p.m./min per mg of protein)
Control	5762 $\pm$ 373
Angiotensin II (100 nM)	12087 $\pm$ 754
CsA	7949 $\pm$ 459*
CsA + angiotensin II (100 nM)	15098 $\pm$ 904**
CsA + nifedipine (1 μ M)	7877 $\pm$ 397
CsA + nifedipine (1 μ M) + angiotensin II (100 nM)	15787 $\pm$ 831

Table 2. Effect of CsA on angiotensin II-stimulated $^{45}\text{Ca}^{2+}$ efflux from smooth muscle cells

The cells were prelabelled with $^{45}\text{Ca}^{2+}$ ($4\ \mu\text{Ci}/\text{ml}$) for 24 h and CsA ($10\ \mu\text{g}/\text{ml}$) was then added for a further 3 h. Cells were then stimulated with angiotensin II ($100\ \text{nM}$) or vehicle. Data are expressed as fractional loss/5 min as outlined under 'Experimental'. The values for the first interval after the addition of angiotensin II or vehicle are indicated. Results are means $\pm$ S.E.M. for $n = 6$. * $P < 0.05$ versus angiotensin II, by Student's t -test.

Addition	Fractional loss of $^{45}\text{Ca}^{2+}$
Control	0.12 ± 0.02
Angiotensin II ($100\ \text{nM}$)	0.35 ± 0.03
CsA	0.13 ± 0.01
CsA + angiotensin II ($100\ \text{nM}$)	$0.48 \pm 0.04^*$

DISCUSSION

The success of organ transplantation has greatly benefited from the use of immunosuppressive drugs, especially CsA. However, serious side-effects of CsA in some of the patients are nephrotoxicity [19] and arterial hypertension [4]. It has been reported that CsA enhances the contractility of vascular smooth muscle [5,6]; this may explain the hypertensive side-effect. As an increase of $[\text{Ca}^{2+}]_i$ is a prerequisite of smooth muscle contraction [7], the aim of this study was to determine if CsA alters the calcium homeostasis in SMC prepared from rat aorta and stimulated with the vasoconstrictive hormone angiotensin II. It is well documented that angiotensin II stimulates phosphoinositide hydrolysis and calcium mobilization in SMC from rat aorta [20–22]. We now report that pretreatment of SMC with CsA increased the amplitude and duration of the angiotensin II-induced rise in $[\text{Ca}^{2+}]_i$. This increase in $[\text{Ca}^{2+}]_i$ induced by angiotensin II appears to reflect: (1) an increase in the hormone-induced mobilization of Ca^{2+} from intracellular stores, and (2) an increased Ca^{2+} influx from the extracellular space.

CsA increased basal Ca^{2+} influx (Table 1) and this may possibly have increased the angiotensin II-sensitive intracellular Ca^{2+} pool, which is assumed to be the sarcoplasmic reticulum [23]. As a consequence, upon addition of angiotensin II an enhanced mobilization of Ca^{2+} from intracellular stores is observed. As CsA also augments the $[\text{Arg}^8]\text{vasopressin}$ -induced rise in $[\text{Ca}^{2+}]_i$ (Figs. 3c and 3g; J. Pfeilschifter & U. T. Regg, unpublished work), the effect of CsA on intracellular calcium homeostasis seems to be a more general phenomenon.

The intracellular mediation of the CsA effects on target cells is generally not well understood. There is evidence for a cytosolic binding protein for CsA named cyclophilin [24]. The dissociation constant for CsA to cyclophilin is $2 \times 10^{-7}\ \text{M}$. Clinical results [25,26] have suggested that optimal plasma levels of CsA for the immunosuppressive effect in man are in the range of $200\ \text{nM}$ – $1\ \mu\text{M}$. We found half-maximal effects of CsA on angiotensin II-induced $[\text{Ca}^{2+}]_i$ rise in rat SMC at about $1.7\ \mu\text{M}$. In this context it is important to know that the rat has been shown to be relatively resistant to the immunosuppressive effects of CsA compared with man

[27]. Thus, a mediation of the effect of CsA on $[\text{Ca}^{2+}]_i$ in SMC by a binding to cyclophilin is not unlikely. However, it is not known in which way binding of CsA to cyclophilin could alter cellular functions.

Recently it has been shown that CsA binds to calmodulin [28] and it has been demonstrated that CsA inhibits calmodulin-dependent reactions ([28], but see also [29]). Moreover, binding of CsA to calmodulin was found to be completely inhibited by calmodulin antagonists. As calmodulin activates inositol 1,4,5-trisphosphate 3-kinase activity in pig aortic smooth muscle [30], CsA might interfere with the activity of this enzyme and thereby alter the metabolism of inositol 1,4,5-trisphosphate as well as that of inositol 1,3,4,5-tetrakisphosphate.

Inositol 1,4,5-trisphosphate is the second messenger for the mobilization of Ca^{2+} from intracellular stores in various cell types (see [23] for review) and has also been suggested to activate voltage-insensitive Ca^{2+} channels in T-lymphocytes [31]. On the other hand, inositol 1,3,4,5-tetrakisphosphate has been suggested as a second messenger controlling cellular Ca^{2+} homeostasis at the plasma membrane [32]. Thus, CsA may prove to be a useful tool to clarify the molecular mechanism underlying the control of opening of non-voltage-dependent Ca^{2+} channels in SMC. The effect of CsA to enhance vasoconstrictor-mediated Ca^{2+} mobilization and contraction in SMC may be a causal factor in CsA-induced arterial hypertension. A detailed knowledge of the molecular mechanism involved in the elevation of $[\text{Ca}^{2+}]_i$ should be helpful in understanding of this side-effect of CsA and may have important therapeutic implications with regard to CsA administration.

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REFERENCES

- Shevach, E. M. (1985) *Annu. Rev. Immunol.* **3**, 397–423
- Orosz, G. G., Roopenian, D. C., Widmer, M. B. & Bach, F. H. (1983) *Transplantation* **36**, 706–711
- Graneli-Piperno, A., Inaba, K. & Steinman, R. (1984) *J. Exp. Med.* **160**, 1792–1802
- Bellet, M., Cabrol, C., Sassano, P., Leger, P., Corvol, P. & Menard, J. (1985) *Am. J. Cardiol.* **56**, 927–932
- Xue, H., Bukoski, R. D., McCaron, D. A. & Bennett, W. M. (1987) *Transplantation* **43**, 715–718
- Lamb, F. S. & Webb, R. C. (1987) *Life Sci.* **40**, 2571–2578
- Sommerville, L. E. & Hartshorne, D. J. (1986) *Cell Calcium* **7**, 353–364
- Nicchitta, C. V., Kamoun, M. & Williamson, J. R. (1985) *J. Biol. Chem.* **260**, 13613–13618
- Hiestand, P. C. (1984) *Agents Actions* **15**, 556–561
- Ross, R. (1971) *J. Cell Biol.* **50**, 172–186
- Travo, P., Weber, K. & Osborn, M. (1982) *Exp. Cell Res.* **139**, 87–54
- Osborn, M. & Weber, K. (1982) *Methods Cell Biol.* **24**, 31–43
- Debus, E., Weber, K. & Osborn, M. (1983) *EMBO J.* **2**, 2305–2312
- Pfeilschifter, J., Kurtz, A. & Bauer, C. (1986) *Biochem. J.* **234**, 125–130

15. Pfeilschifter, J. & Bauer, C. (1986) *Biochem. J.* **236**, 289–294
16. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–344
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
18. Nowicky, M. C., Fox, A. P. & Tsien, R. W. (1985) *Nature (London)* **316**, 440–443
19. Ryffel, B., Siegl, H., Thiel, G. & Mihatsch, M. J. (1986) in *Kidney Transplant Rejection* (Williams, G. M., Burdick, J. F. & Solez, K., eds.), pp. 383–410, Marcel Dekker, New York and Basel
20. Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S. & Farese, R. V. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7812–7816
21. Capponi, A. M., Lew, P. D. & Vallotton, M. B. (1985) *J. Biol. Chem.* **260**, 7836–7842
22. Alexander, R. W., Brock, T. A., Gimbrone, M. A., Jr. & Rittenhouse, S. E. (1985) *Hypertension* **7**, 447–451
23. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
24. Handschumacher, R. E., Harding, M. W., Rice, J. & Drugge, R. J. (1984) *Science* **226**, 544–547
25. Kahan, B. D. (1982) *Clin. Haematol.* **2**, 743–761
26. Griffith, B. P., Hardesty, R. L., Trento, A., Lee, A. & Bakson, H. T. (1984) *J. Thorac. Cardiovasc. Surg.* **88**, 952–957
27. Whiting, P. H., Thompson, A. W., Blair, J. T. & Simpson, J. G. (1982) *Br. J. Exp. Pathol.* **63**, 88–94
28. Colombani, P. M., Robb, A. & Hess, A. D. (1985) *Science* **228**, 337–339
29. LeGrue, S. J., Turner, R., Weisbrodt, N. & Deman, J. R. (1986) *Science* **234**, 68–71
30. Yamaguchi, K., Hirata, M. & Kuriyama, H. (1987) *Biochem. J.* **244**, 787–791
31. Kuno, M. & Gardner, P. (1987) *Nature (London)* **326**, 301–304
32. Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920

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