Identification of a set of calcium-binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum

DARRYL R. J. MACER and GORDON L. E. KOCH
Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Summary

A procedure was developed for the isolation of reticuloplasm, the luminal material of the endoplasmic reticulum (ER). A reticuloplasm-rich extract was prepared from a murine plasmacytoma cell line that contains large amounts of ER, by first extracting the cytoplasmic contents using hypotonic lysis to yield ER-rich 'shells' followed by mechanical lysis to release the ER contents. The extract contains five major proteins with apparent molecular weights of 100, 75, 60, 58 and 55 (10^3) Mr by SDS–polyacrylamide gel electrophoresis. The 100, 75 and 58 (10^3) Mr species were identified as the known ER proteins endoplasmin, BiP and PD1, respectively. The ER association of the 60 and 55 (10^3) Mr proteins was confirmed by confocal fluorescence microscopy with affinity-purified antibodies.

A calcium overlay test revealed that, in addition to endoplasmin, reticuloplasm contained at least three other calcium-binding proteins: i.e. BiP, PD1 and the 55×10^3 Mr protein, respectively, with endoplasmin and the 55×10^3 Mr protein (CRP55) accounting for the major proportion of the calcium-binding activity.

Treatment of cells with calcium ionophore led to the specific over-expression of the major calcium-binding reticuloplasmins endoplasmin, BiP and CRP55.

These studies show that the lumen of the ER contains a family of proteins with the capacity to bind significant amounts of calcium in the millimolar range and thereby to confer upon the ER the ability to perform a calcium storage function analogous to that of the sarcoplasmic reticulum in muscle cells.

Key words: endoplasmic reticulum, reticuloplasm, calcium-binding proteins, endoplasmin, BiP, protein disulphide isomerase, CRP55.

Introduction

Calcium ions play a vital role in a variety of cellular processes. This is achieved through a stringent control of calcium levels within cells (Carafoli, 1987; Somlyo, 1984). One of the major mechanisms for maintaining intracellular calcium in the sub-micromolar range is the Ca^{2+}–ATPase system, which pumps calcium actively out of the cytoplasm through the plasma membrane (Campbell, 1983). However, it is also known that intracellular membrane systems can sequester significant amounts of calcium and release this for use in intracellular signalling (Hasselbach & Makinose, 1961; Somlyo, 1984; Baker, 1986; Streb et al. 1983). One of the best-characterized intracellular systems operating under normal physiological conditions is the sarcoplasmic reticulum (SR) in muscle, which has been clearly established as the system for removing Ca^{2+} from muscle cytoplasm to levels that would induce dissociation from troponin (MacLennan & Holland, 1975). It is now widely accepted that in non-muscle systems the mitochondria play a relatively minor role in calcium homeostasis and attention has been focussed on the role of the endoplasmic reticulum (ER) in the regulation of cytosolic calcium levels (Henkart, 1975; Somlyo, 1984; Baker, 1986; Streb et al. 1983; Berridge & Irvine, 1984). In this respect it has been suggested that the ER might be able to store and release calcium in a manner analogous to that involved in the sarcoplasmic reticulum (Baker, 1986). Thus, it has been shown directly that the calcium concentration within the ER is relatively high and that the calcium can be released by agents such as inositol triphosphate and GTP (Streb et al. 1984; Gili et al. 1986). Isolated microsomes can accumulate calcium in an
ATP-dependent process and release it under the influence of the above-mentioned agents (Spat et al. 1987). The existence of a Ca^{2+}-ATPase activity capable of pumping calcium into the ER has also been suggested (Moore & Klaus-Freedmann, 1983). However, one major deficiency in this area is a lack of knowledge about the nature of the stored calcium in the ER. By analogy with SR one would expect the ER in non-muscle cells to contain one or more proteins in the luminal space or reticuloplasm that could carry out a role analogous to that of calsequestrin (MacLennan & Holland, 1975). Indeed, it has recently been established that the reticuloplasm is a protein-rich medium that contains a family of abundant resident proteins called reticuloplasmins (Koch, 1987), one of which is a major glycoprotein called endoplasm, which is able to bind significant amounts of calcium in the millimolar range (Koch et al. 1986a).

In this study we have carried out a systematic study of reticuloplasm with a view to identifying the repertoire of calcium-binding proteins associated with the ER. To achieve this, we have developed a general procedure for isolating reticuloplasm, identified and characterized the major abundant proteins in the isolate and examined these for calcium-binding activity.

**Materials and methods**

**Growth and preparation of cells**

MOPC-315 cells (Eisen et al. 1968) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, 100 000 units ml^{-1} penicillin/streptomycin and 4 mM-pyruvate to a density of ~5X 10^5 cells ml^{-1}, harvested by centrifugation at 3000g and washed thoroughly with phosphate-buffered saline (PBS).

**Preparation of reticuloplasm**

Washed MOPC-315 cells were suspended at 1X 10^7 cells min^{-1} in 10 mM-Tris-HCl, pH 7.5, on ice and allowed to swell and lyse completely. The resultant 'shells' were centrifuged out at 10000 g, suspended in PBS at 1X 10^6 cells equivalent per ml and disrupted by syringing through an 18 gauge needle. The cell debris was pelleted by centrifuging at 100 000 g for 30 min. The supernatant was collected and protease inhibitors (Koch et al. 1986a) added. The supernatant was stored at 4°C. For longer storage at -20°C glycerol was added to 50% to prevent freezing, and dialysed out before use.

**Preparation of sarcoplasmic reticulum vesicles**

The method used was based on that of MacLennan (1970), and was used to obtain vesicles from rabbit and murine skeletal muscle. Muscle tissue was homogenized in an equal volume of 120 mM-NaCl, 5 mM-imidazole, pH 7.4. It was spun at 1000 g for 5 min, and the pellet was re-extracted with buffer. The combined superantants were squeezed through four layers of muslin, and the pH adjusted to 7.4. The suspension was spun at 10 000 revs min^{-1} in a SS-34 rotor for 15 min. The supernatant was squeezed through four layers of muslin, and centrifuged at 16 000 revs min^{-1} in a SS-34 rotor for 30 min. The pellet is the preparation of sarcoplasmic reticulum vesicles.

**Purification of calsequestrin**

Sarcoplasmic reticulum vesicles were suspended in PBS and saponin was added to a final concentration of 0.01%. After 10 min on ice the membranes were centrifuged out at 100 000 g for 50 min. The supernatant contains calsequestrin at >95% purity.

**Preparation of antibodies to individual reticuloplasmins**

Rabbits (Dutch White or New Zealand White) were immunized with 1–2 mg of purified reticuloplasmin. The first injection was with Freund’s complete adjuvant and subsequent injections were at monthly intervals without adjuvant. Serum was collected by standard procedures and stored in 0.02% sodium azide.

Monospecific antibodies to particular proteins in the purified reticuloplasmin were prepared, as described (Koch et al. 1986a). A sample of reticuloplasmin was fractionated on a preparative SDS–polyacrylamide gel and the proteins were transferred to a...
Fig. 2. Monospecific antibodies to the major reticuloplasmins from MOPC-315 cells. Antibodies were prepared as described in Materials and methods, and tested by immunoblotting analysis on two-dimensional gels of lysates from MOPC-315 cells (the samples had been partially depleted for endoplasmin (Koch et al. 1986)) with ConA–agarose). Panel L shows the protein pattern from the lysate; and panels EP, BiP, P60, PDI and P55 show immunoblots developed with antibodies to endoplasmin, BiP, P60, PDI and CRP55, respectively (see text).

nitrocellulose sheet by electroblotting (see below). The nitrocellulose was stained with 0.01% Ponceau Red in 5% acetic acid and the strips corresponding to each protein component were carefully excised. The strips were then immersed in the undiluted immune serum and mixed by rotation at 4°C for at least 24 h. The strips were removed, washed thoroughly with PBS and the antibodies eluted with 0.2 M- glycine–HCl, pH 2 (10 min at 4°C). The eluate was immediately neutralized to pH 7.5 with 1 M-Tris–base and tested for antibodies by immunoblotting on cell lysates. Strips can be used repeatedly for the extraction of antibody from unfractionated serum.

Gel electrophoresis and immunoblotting
SDS–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) and two-dimensional gel electrophoresis by the method of O’Farrell (1975). Immunoblotting was as described by Towbin et al. (1979). Rabbit antibodies were developed with 125I-labelled Protein A (Amersham).

Immunofluorescence microscopy
Immunofluorescence microscopy was carried out as described (Koch et al. 1987) using the confocal microscope described by White et al. (1987). MOPC-315 cells were fixed in 3.5% formaldehyde in PBS (5 min), permeabilized with 1% Nonidet P40 (5 min), labelled with the appropriate antibody (5 min) and developed with fluorescein-conjugated goat anti-rabbit Ig.

Purification of proteins from reticuloplasmin
Endoplasmin was selectively extracted from purified reticuloplasmin with Con A–Sepharose as described (Koch et al. 1986). The non-bound fraction was dialysed against 10 mM-Tris·HCl, pH 7.5, and made up to 50% saturation with solid ammonium sulphate. The precipitate was centrifuged out and the supernatant made up to 85% saturation with solid ammonium sulphate. The precipitate was collected by centrifugation, dissolved in 10 mM-Tris·HCl, pH 7.5, and dialysed against the same buffer. The sample was applied to DEAE-Sephadex equilibrated with 10 mM-Tris·HCl, pH 7.5, and eluted with a linear gradient (10 mM to 70 mM-Tris·HCl, pH 7.5). Fractions were monitored by SDS–polyacrylamide gel electrophoresis and immunoblotting and those containing (in order of elution) P58, BiP, PDI and CRP55 were pooled and concentrated. Each sample was fractionated by gel filtration on Sephacryl S200 to

Calcium-binding reticuloplasmins
Fig. 3. Immunofluorescence localization of the major reticuloplasmins from MOPC-315 cells. Samples were prepared and stained, as described (Koch et al. 1987). A: Endoplasmin; B, P60; C, PD1; D, CRP55.

give the purified proteins. All of the purified preparations were >90% pure for the respective protein (Macer, 1988).

**Results**

**Purification of reticuloplasm**

The approach used for the isolation of the luminal contents of the ER, referred to as reticuloplasm, was based on previous studies on the major glycoprotein endoplasmin (Koch et al. 1986a, 1987). We have previously established (Koch et al. 1988) that endoplasmin is a major component of reticuloplasm and can therefore be used a marker for the isolation of the contents of the ER. It was found that the exposure of murine plasmacytoma cells (in this study we used the MOPC-315 line but any plasmacytoma cell line can be employed) to hypotonic conditions resulted in the extraction of all the cytoplasmic proteins, but endoplasmin itself was retained in the shells produced by the lysis. Fig. 1 shows that, in addition to endoplasmin, the major 100×10^3 M_r protein in such preparations, several other major proteins are selectively retained in the shells. When the shells were disrupted mechanically by repeated syringing through a hypodermic needle and the particulate material was pelleted by high-speed centrifugation, the supernatant material contained five major proteins with apparent molecular weights (on SDS–polyacrylamide gels) of 100, 75, 60, 58 and 55(×10^3) M_r, respectively.

**Characterization of the major proteins in isolated reticuloplasm**

The major proteins in the soluble extract from the shells
obtained from the plasmacytoma cells were characterized by determining the amino-terminal sequence of each. For this, each protein was purified by electroblotting onto PVDF membranes and the amino-terminal sequence was determined by the procedure described by Matsudaira (1987). The sequences obtained (Macer, 1988) confirmed that the $100 \times 10^3 M_r$ protein is endoplasmic and show that the $75 \times 10^3 M_r$ protein is another previously identified ER-associated protein, the so-called immunoglobulin heavy-chain binding protein (BiP) (Lee et al. 1984; Munro & Pelham, 1987). The 60 and $55(\times 10^3) M_r$ proteins have amino-terminal sequences that are not identifiable with any known sequence in the major sequence libraries. The $58 \times 10^3 M_r$ protein did not yield any amino-terminal sequence, suggesting that it might be blocked. However, it was identifiable as the previously identified microsomal protein, protein disulphide isomerase (PDI) by immunoblotting with a monospecific antibody (kindly provided by Dr R. Freedman).

The ER location of the five major putative reticuloplasmins was confirmed by immunofluorescence microscopy. Monospecific antibodies to each protein were prepared by the nitrocellulose affinity strip procedure described (Koch et al. 1986a). Briefly, rabbits were immunized with the whole reticuloplasm extract. Nitrocellulose strips containing each protein were prepared, by running a preparative SDS-polyacrylamide gel on the reticuloplasm extract, electroblotting onto nitrocellulose and cutting out the strip bearing each band, and used to purify antibodies to each protein. The specificity of each antibody was confirmed by immunoblotting on whole cell lysates from the plasmacytoma cell line (Fig. 2). When used in immunofluorescence tests on plasmacytoma cells with the confocal fluorescence microscope, all antibodies gave the same pattern characteristic of the endoplasmic reticulum of plasmacytoma cells (Fig. 3). The result for BiP is not shown, since its ER localization has been established (Munro & Pelham, 1987).

These studies confirm that all the major proteins in the sample of isolated reticuloplasm are reticuloplasmins, i.e. luminal proteins of the ER, and justify the use of this material for the study of the calcium-binding properties of reticuloplasm.

**Calcium-binding capacity of isolated reticuloplasm**

Equilibrium dialysis experiments were used to examine the calcium-binding capacity of isolated reticuloplasm. These experiments were carried out in 10 mm-Tris-HCl, pH 7.5, with 100 mM-KCl, since this is the standard...
Ca Pr

Fig. 6. Identification of calcium-binding proteins in enriched reticuloplasm. Enriched reticuloplasm was purified, fractionated on two-dimensional polyacrylamide gels (O'Farrell, 1975), transferred to nitrocellulose (Towbin et al. 1979) and developed with $^{45}$Ca (Koch et al. 1986). The name spots were identified with the corresponding monospecific antibodies (Fig. 2). The sample was partially depleted for endoplasmin with ConA-agarose (Koch et al. 1986a). Spots 1, endoplasmin; 2, BiP; 3, PD1; 4, CRP55.

buffer used in the study of calcium-binding by sarcoplasmic reticulum vesicles and the major calcium-storage protein calsequestrin (Campbell et al. 1983). Fig. 4 shows that calcium-binding saturates in the millimolar range, half-maximal binding occurring at about 2.5 mM-calcium. At 10 mM-calcium the isolated reticuloplasm binds ~300 nmoles calcium per mg protein.

Previous studies have shown that endoplasmin is one of the major calcium-binding proteins in plasmacytoma cells (Koch et al. 1986a). However, the binding studies were not performed under equilibrium conditions so they were repeated here using equilibrium dialysis, as described above. At 5 mM-calcium native endoplasmin bound ~280 nmoles calcium per mg protein, supporting the previous suggestions that endoplasmin is a major calcium-binding protein of the ER.

Calcium-binding proteins in reticuloplasm

The repertoire of calcium-binding proteins in reticuloplasm was examined by the SDS–polyacrylamide gel/electroblotting/$^{45}$Ca overlay technique used previously (Koch et al. 1986b). The advantage of this technique, apart from its intrinsic convenience, is that it can be used on complex mixtures as well as pure samples of proteins (Fig. 5) and with a sample of purified sarcoplasmic reticulum vesicles (Fig. 5). In the synthesis mixture, significant calcium-binding is observed with only the known calcium-binding protein α-lactalbumin. In the sample of SR vesicles, the major calcium-binding species has an apparent molecular weight of ~55×10^3 and co-migrates with pure calsequestrin, which itself binds substantial amounts of calcium under the conditions used. We therefore conclude that the $^{45}$Ca overlay technique was suitable for the detection of calcium-binding proteins of the calsequestrin-type, that is, high-capacity and low-affinity.

Fig. 5 shows that there are several calcium-binding proteins in a whole cell lysate derived from the plasmacytoma cell. The major binding species have apparent molecular weights of 100, 90, 75, 58 and 55×10^3, respectively. Fig. 5 shows that the 100, 75, 58 and 55(×10^3) M, calcium-binding species co-purify with re-
cytoplasmic calcium-binding protein, shows saturable binding even at millimolar concentrations. Gelsolin, which is a sarcoplasmic reticulum protein, calsequestrin. (•), human gelsolin; (D), murine calsequestrin; (■), murine CRPS5; (□), human transferrin.

**Fig. 7.** Concentration dependence of calcium-binding proteins in the $^{45}$Ca overlay assay. The assay was carried out, as described in Materials and methods. (•), Rabbit calsequestrin; (■), human gelsolin; (□), murine endoplasm; (Δ), murine CRP55; (○), human transferrin.

ticuloplasm. Two-dimensional gel analysis (Fig. 6) showed that the major calcium-binding proteins in reticuloplasm were endoplasm, BiP, PDI and the $55 \times 10^3 M_r$ reticuloplasmin, respectively.

Sufficient quantities of the proteins, apart from endoplasm, were not available for examining their calcium-binding capacities by equilibrium dialysis. However, the nitrocellulose binding test was adapted to permit a comparison with calsequestrin under non-equilibrium conditions. Briefly, small amounts of purified non-denatured protein were spotted out on nitrocellulose filters and incubated with varying concentrations of $^{45}$Ca, the filters briefly washed and each sample counted to measure calcium binding. Fig. 7 shows the binding profiles for several proteins obtained in this assay. Proteins like transferrin do not bind significant amounts of calcium even at millimolar concentrations. Gelsolin, which is a cytoplasmic calcium-binding protein, shows saturable binding in the region of 10$\mu$M-calcium. However, it is clear that low-affinity/high capacity calcium-binding proteins such as calsequestrin can be distinguished from the high-affinity cytoplasmic calcium-binding proteins, such as gelsolin, by this assay. Fig. 7 also shows the binding profiles for endoplasm and the $55 \times 10^3 M_r$ protein and confirms that they resemble calsequestrin with respect to both affinity and capacity. Thus, although the assay has the limitation of a non-equilibrium analysis, it does indicate that the binding characteristics of the calcium-binding reticuloplasmins are similar to those of the sarcoplasmic reticulum protein, calsequestrin.

**Effect of calcium ionophore on the protein composition of plasmacytoma cells**

Fig. 8 shows that calcium ionophore has a specific effect on the protein composition of MOPC-315 cells. Even at the level of one-dimensional SDS-polyacrylamide gel electrophoresis, at least three protein bands are increased significantly and these bands co-migrate with endoplasm, BiP and the $55 \times 10^3 M_r$ reticuloplasmin. Immunoblotting studies (Fig. 8) confirm that these three proteins are indeed over-expressed in cells treated with calcium ionophore. In contrast, PDI and the $60 \times 10^3 M_r$ reticuloplasmin are not increased by ionophore (Macer, 1988). Studies on the kinetics of this overexpression show that, as observed previously for endoplasm (GRP95) and BiP (GRP75) (Lee et al. 1984; Welch et al. 1983), the increased expression of these proteins is apparent only after 3–4 h in the ionophore and reaches a maximum in 12–16 h. These studies extend the previous observations that endoplasm and BiP are over-expressed in calcium ionophore-treated cells, by showing that a third protein, the $55 \times 10^3 M_r$ reticuloplasmin (referred to as CRP55 to emphasize its calcium-binding and calcium-regulated characteristics), it also over-expressed in such cells, and also show that all three proteins specifically increased in these cells are prospective calcium-binding proteins in the reticuloplasm.

**Discussion**

The purpose of the present study was to determine whether reticuloplasm, the luminal material of the endoplasmic reticulum (Krstic, 1979; Koch, 1987), contains proteins that could serve the calcium-storage function performed by calsequestrin in the sarcoplasmic reticulum. To this end, we developed a simple procedure for isolating reticuloplasm from a cultured cell line. The choice of the plasmacytoma cell was determined by the fact that it contains large amounts of endoplasmic reticulum. Consequently, when the cells are perforated by hypotonic lysis and the cytoplasmic contents released, the resultant shells are essentially ER and nuclei only. Fortunately, when these hypotonic shells are disrupted mechanically, the contents of the ER are released in a soluble form and are easily obtained in a highly purified state. This was confirmed directly by showing that all the major proteins in the extract are located in the endoplasmic reticulum. It is worth noting here that shells of very similar composition can be obtained by perforating the same cells with low concentrations of saponin under isotonic conditions. However, mechanical disruptions of such shells does not release the contents of the ER but rather generates closed vesicles that retain the reticuloplasm. These can be released from such preparations only with stronger detergents. Thus, in order to obtain reticuloplasm without resorting to the use of detergents, it is preferable to use the hypotonic lysis procedure.

Equilibrium dialysis studies on isolated reticuloplasm clearly showed that it has a significant calcium-binding capacity. Calsequestrin, under identical conditions, binds 250–800 nmoles of calcium per mg protein and...
In the case of reticuloplasmin, half-maximal binding is at 2.5 mM and at 10 mM-calcium the binding capacity is 300 nmoles per mg protein. Thus, the calcium-binding activity in reticuloplasmin is comparable to that of calsequestrin. The implication is that reticuloplasmin contains proteins that have the ability to bind calcium with high capacity in the millimolar range. However, in preliminary studies with an antibody to calsequestrin, no evidence was obtained for a calsequestrin-like protein associated with the ER itself. Therefore, it was necessary to determine which proteins actually performed the calcium-binding function in reticuloplasmin.

The procedure used to identify the calcium-binding proteins in reticuloplasmin was to fractionate the reticuloplasmin by gel electrophoresis, transfer the proteins to nitrocellulose and probe directly for calcium-binding with 45Ca. This approach has been used previously to identify proteins that bind calcium in the micromolar range (Koch et al. 1986a,b; Maruyama et al. 1984). However, using calsequestrin as a model, we have shown that the method also works for low-affinity/high-capacity calcium-binding proteins. This is probably not surprising, since the calcium-binding sites in such proteins appear to consist of clusters of negative charges, rather than any specific fold, such as the E-F hand found in the high-affinity calcium-binding proteins. It is also worth emphasizing that the test is quite specific, in that it clearly distinguishes between proteins that are known to bind significant amounts of calcium and those that do not. In the case of endoplasmin it was directly confirmed by equilibrium dialysis that the protein does bind significant amounts of calcium under the conditions used in the calsequestrin binding assay. In view of this, we conclude that endoplasmin, BiP, PDI and CRP55 are all significant calcium-binding proteins of the reticuloplasmin. Of these, CRP55 appears to have a similar calcium-binding capacity and affinity to calsequestrin, whilst endoplasmin is of moderate capacity. BiP and PDI bind smaller amounts of calcium but their capacity appears significant enough to make a contribution to the total calcium-binding capacity of reticuloplasmin.

The main conclusion to emerge from these studies is that the lumen of the ER does contain a number of proteins that could participate in the calcium storage.
function attributed to this organelle in non-muscle cells. Since the proteins concerned are known to exist in the ER of most, and probably all, eukaryotic cells (Macer, 1988), it follows that the ER can function as a general calcium storage organelle.

The obvious question that arises now concerns the demonstration that the proteins actually perform the storage function in vivo. This is not to say it is easy and even in the case of calsequestrin, it is based only on circumstantial evidence. However, at least one line of evidence does exist for this in the case of the ER proteins. It has been known for some time that calcium ionophores have a very specific effect on the protein composition of cells. Previous studies showed that endoplasm and BiP were over-expressed in ionophore-treated cells (Lee, 1987). In this study, we have confirmed that a third protein, the $55 \times 10^3 M_r$ reticuloplasm identified in this study (CRP55), is also over-expressed in ionophore-treated cells. Thus, the specific effect of calcium metabolism in cells is to induce the increased production of three ER proteins that have the capacity to bind calcium. It is reasonable to speculate that such a response suggests a role for the calcium-binding, calcium-regulated proteins in calcium homeostasis. One possibility is that the response leads to an increase in the intrinsic calcium storage capacity of the ER and thereby permits the cell to counteract the damaging effects of increased cytosolic calcium caused by the ionophore. Such an explanation is also consistent with the established phenomenon that a variety of stresses can also induce the synthesis of the calcium-binding reticuloplasmins. It is believed that one of the common events following the exposure of cells to stress is an alteration in calcium levels in the cytosol (Trump et al. 1981). If not corrected, this can lead to cell death. The observed increased synthesis of the prospective calcium storage proteins in the ER could be one way of preventing this calcium-induced catastrophe.

In this study, we have been concerned with the question of whether the ER lumen does contain proteins that could carry out a calcium-storage function and we conclude that it does. However, it should be emphasized that, unlike the sarcoplasmic reticulum, the ER performs several other functions and the function of the relevant calcium-binding proteins may not be confined to calcium-storage.

References


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Note added in proof

45Ca overlay experiments using nitrocellulose filters were carried out in 25 mM-Hepes, pH 7.2, 100 mM-KCl, 10 mM-MgCl2, using the procedures described by Koch et al. (1986a).