Volume 328, number 3, 296–300 FEBS 12844 August 1993

Strontium binding to sarcoplasmic reticulum Ca^{2+}-ATPase

Spectroscopic differentiation of the substeps involved

Stéphane Orlowski and Philippe Champeil

Unité de Recherche Associée 1290 (Centre National de la Recherche Scientifique) and Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire (Commissariat à l'Energie Atomique), Centre d'Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France

Received 9 June 1993

We investigated the consequences of Sr^{2+} binding to the transport sites of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase for two fluorescent conformational probes located in different regions of the ATPase. Using SR vesicles in which Lys-515 in the ATPase had been previously labeled with fluorescein 5′-isothiocyanate (FITC), we found that the Sr^{2+}-induced a drop in the fluorescein fluorescence of this FITC-labeled ATPase shifted toward lower Sr^{2+} concentrations than the Sr^{2+}-induced rise in Trp fluorescence for the same FITC-labeled ATPase. The curve describing the Sr^{2+}-dependent rise in Trp fluorescence had a characteristic asymmetric shape, and the changes in Trp fluorescence occurred in parallel with the activation by Sr^{2+} of pNPP hydrolysis by the ATPase. Analysis of these results in terms of the simplest scheme describing the sequential binding of the two Sr^{2+} ions suggests that under the conditions of these experiments, i.e. at neutral pH in the presence of potassium, the Sr^{2+}-induced rise in the Trp fluorescence mainly reflected the formation of ATPase with two ions bound to the transport sites, whereas the binding of a single Sr^{2+} ion was virtually sufficient to reduce the fluorescence of bound FITC to its minimal level.

Sarcoplasmic reticulum; Ca^{2+}-ATPase; Strontium; Fluorescent probe

1. INTRODUCTION

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, the membranous enzyme responsible for the removal of Ca^{2+} from the cytoplasm of muscle cells, is an actively studied member of the family of the P-type ion-transport ATPases [1–3]. Yet, the detailed events leading to ATPase activation by Ca^{2+} are still controversial [4–6]. Ca^{2+}-ATPase activation requires the binding of two Ca^{2+} ions to its transport sites, but the high degree of positive cooperativity generally believed to exist for Ca^{2+} binding [7] makes it difficult to design equilibrium experiments that would discriminate between the effects of the binding of either one or two Ca^{2+} ions.

Correspondence address: S. Orlowski, Unité de Recherche Associée 1290 (Centre National de la Recherche Scientifique) and Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire (Commissariat à l'Energie Atomique), Centre d'Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France. Fax. (33) (1) 6908-8139.

Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid, Tris, tris(hydroxymethyl)aminomethane; FITC, fluorescein 5′-isothiocyanate; Trp, tryptophan; Lys, lysine; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; A23187, calcimycin; pNPP, *p*-nitrophenylphosphate; E, EM, EM_2, ATPase species with zero, one or two Sr^{2+} ions bound to the transport sites.

In addition to Ca^{2+}, the sarcoplasmic reticulum Ca^{2+}-ATPase may transport Sr^{2+} [8–12]. Although the ATPase has a poorer affinity for Sr^{2+} than for Ca^{2+}, the stoichiometry of two ions transported per ATP molecule hydrolyzed was found to be the same in both cases [13]. Yet, it has been repeatedly suggested that Ca^{2+}-ATPase activation by Sr^{2+} was less cooperative than its activation by Ca^{2+} [14–15]. Binding of $^{90}Sr^{2+}$ itself has never been directly measured, because of the technical difficulties involved in measuring the binding of a poor-affinity ligand. Nevertheless, from indirect rapid mixing experiments, Fujimori and Jencks recently concluded that Sr^{2+} binding occurred with poor cooperativity. Binding constants under neutral conditions were estimated to be 35 μM and 55 μM for binding of the first and the second Sr^{2+} ion, respectively [16].

Various fluorescence probes of SR Ca^{2+}-ATPase, including the intrinsic tryptophan residues (Trp) located in the transmembrane section of Ca^{2+}-ATPase, as well as fluorescein 5′-isothiocyanate (FITC) specifically bound to Lys-515 in the nucleotide binding region, have proved useful to monitor the events associated with ion binding to the ATPase transport sites [14–15, 17–21]. However, these probes are usually used as 'black boxes', as the precise events to which they respond are often a matter of conjecture. In this work, we took advantage of the poor cooperativity of Sr^{2+} binding to the ATPase to carefully compare how Trp residues and bound FITC

 Published by Elsevier Science Publishers B.V.

in FITC-labeled ATPase responded to Sr^{2+} binding. As extrinsic probes are a priori suspected to report on proteins with properties different from those of the unlabeled proteins studied with the intrinsic probes, the behavior of the ATPase Trp residues was examined using FITC-labeled vesicles, thus enabling us to compare the response to Sr^{2+} of two probes of the same labeled protein. We found that FITC labeling did not alter the Sr^{2+} sensitivity of the ATPase Trp residues, and that, using FITC-labeled vesicles, the two conformational probes of the same ATPase molecule depended on the free Sr^{2+} concentration in different ways. This finding opens the way toward future characterization of the events to which each probe responds: as Sr^{2+}-induced changes in the fluorescein fluorescence shift toward lower Sr^{2+} concentrations than the Sr^{2+}-induced changes in the Trp fluorescence of the same FITC-labeled ATPase, our results suggest that the FITC fluorescence level drops at an early stage during the sequential process leading to binding of the two ions to the ATPase transport sites, whereas the fluorescence of the Trp residues mainly rises at a subsequent stage.

2. EXPERIMENTAL

Experimental procedures concerning SR vesicle preparation and labeling, as well as fluorescence experiments, were identical to those previously described [22–23]. pNPP hydrolysis by the ATPase was monitored through the optical density changes observed at 420 nm [24]. To estimate the free metal concentrations in the various metal–EGTA mixtures at pH 7.0, we used the following apparent dissociation constants: 3.8×10^{-7} M for Ca-EGTA, 34×10^{-3} M for Mg-EGTA, and 1×10^{-4} M for Sr-EGTA [25–26]. For the experiment illustrated in Fig. 3, to reliably buffer the Ca^{2+} concentration in the 2–20 μM range, we used a Mg/EDTA buffer, whose affinity for Ca^{2+} was 62 μM under these conditions [27].

3. RESULTS

Fig. 1 illustrates an experiment in which FITC-labeled vesicles were suspended in a standard pH 7 medium containing 100 mM KCl and 5 mM Mg^{2+}. As the excitation and emission spectra of fluorescein and tryptophan do not interfere with each other, it was possible to measure both the FITC fluorescence and the Trp fluorescence of the ATPase in these FITC-labeled vesicles; it was therefore possible to monitor the changes experienced by the two different probes of the same protein after a particular addition to the cuvette. The Ca^{2+} chelator EGTA was added first. As described by many groups, the chelation of endogenous and contaminating Ca^{2+} was accompanied by a fall in Trp fluorescence and by a rise in FITC fluorescence. Sr^{2+} was then added, at various concentrations, and these sequential additions reversed the fluorescence changes experienced by Trp residues and bound FITC. Remarkably, however, the fluorescence level of bound FITC was proportionally more sensitive to small concentrations of Sr^{2+} than that of Trp residues. Such traces can be easily

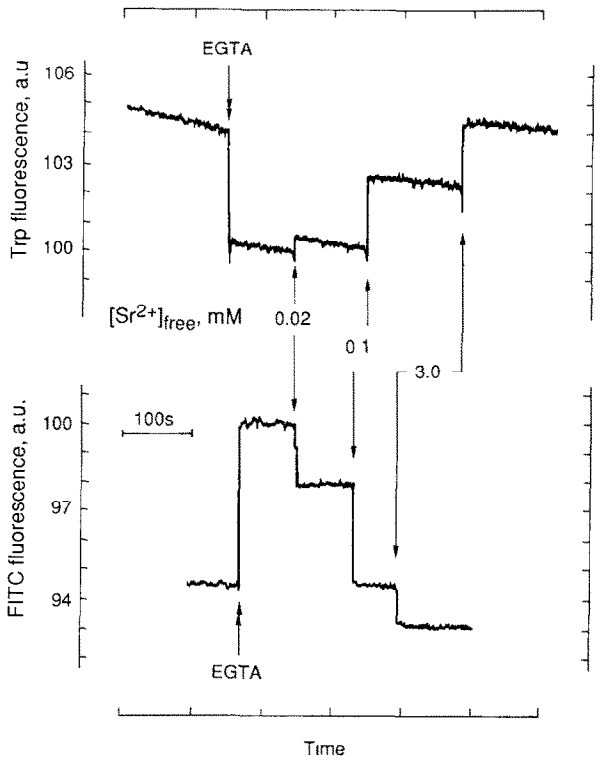

Fig. 1. Different sensitivities to small Sr^{2+} concentrations shown by Trp residues and bound FITC in FITC-labeled SR vesicles. In two successive experiments, FITC-labeled SR vesicles (0.1 mg/ml protein) were initially suspended in 2 ml of a medium containing 50 mM MOPS-Tris, 100 mM KCl, 5 mM $MgCl_2$ and contaminating Ca^{2+} (pH 7, 20°C). In one of the experiments, Trp fluorescence was monitored (upper trace; the excitation and emission wavelengths were 290 and 345 nm respectively); in the other, FITC fluorescence was monitored (lower trace; the excitation and emission wavelengths were 480 and 520 nm respectively). In both cases, 1.2 mM EGTA was first added to the FITC-labeled SR. Sequential addition of small volumes of concentrated Sr^{2+} solutions was then performed, resulting in total Sr^{2+} concentrations of 0.2 mM, 0.7 mM and 4.1 mM. The resulting free Sr^{2+} concentrations are indicated on the figure. Traces were corrected for the (very small) changes due to dilution

recorded in a single series of experiments, by changing the fluorimeter optical settings and amplification levels, but leaving everything else identical in the sample cuvette, thus making any possibility that this observation might be artefactual difficult to substantiate.

Fig. 2 quantifies the Sr^{2+} dependence of the Trp (open triangles) and fluorescein (closed triangles) fluorescence of FITC-labeled ATPase. Two features are immediately apparent, demonstrating that the two probes responded to different events: (i) at low Sr^{2+} concentrations, the Trp fluorescence level was virtually unaltered by Sr^{2+} concentrations which reduced the fluorescein fluorescence level significantly, as was shown in Fig. 1; (ii) the apparent ATPase affinity for Sr^{2+} revealed by the Trp signal was poorer than that revealed by the fluorescein signal; midpoints were 80–90 and 40–50 μM respectively for the two curves.

The Sr^{2+}-dependence of the Trp fluorescence of unlabeled SR vesicles was virtually identical to that of

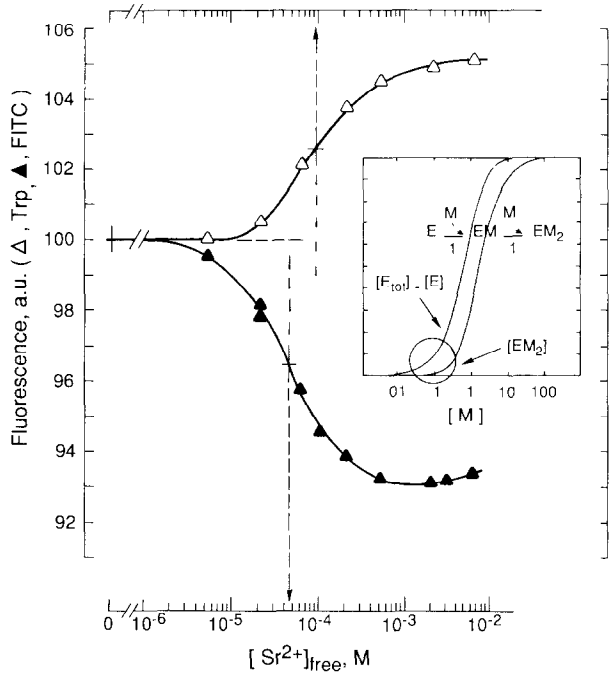

Fig. 2. Parallel measurements of Trp and FITC fluorescence in FITC-labeled SR vesicles, as a function of free Sr^{2+}. Main frame: experimental conditions were identical to those described in the legend to Fig. 1. Open symbols, Trp; closed symbols, fluorescein. Fluorescence is expressed in arbitrary units (a.u.). Inset: theoretical simulation of a sequential binding mechanism with identical dissociation constants for the two steps. The constants were taken as 1, in arbitrary concentration units. The right-hand curve is the proportion of ATPase with 2 ions bound ($[EM_2]$); the left-hand curve is the complement to the proportion of ATPase molecules with no bound ions ($[E_{tot}]-[E]$); the curve describing the average number of bound ions, $(2[EM_2]+[EM])$, would have an intermediate shape, with a Hill coefficient of 1.33, and would be symmetric with respect to its midpoint (not shown).

FITC-labeled vesicles. When we carefully measured this Sr^{2+}-dependence (triangles in Fig. 3A), the titration curve displayed a peculiar asymmetric shape (see also [15]). The latter can be simply appreciated by visual inspection, by comparing the free Sr^{2+} concentrations allowing 10% or 90% of the total fluorescence changes to develop: these concentrations are asymmetrically situated on each side of the midpoint. On the corresponding Hill plot (not shown) the slope was relatively high in the initial portion of the curve, but was closer to 1 in the latter portion, i.e. at high Sr^{2+} concentrations.

Using pNPP as the ATPase substrate in the same series of experiments, we also monitored the activation, by Sr^{2+}, of the ATPase hydrolytic activity (triangles in Fig. 3B). The midpoint for the activation of pNPPase activity was similar to that for the Trp rise (70–80 μM). As a complementary test, Ca^{2+} was used (circles) instead of Sr^{2+}, and both Trp fluorescence (panel A) and pNPPase activity (panel B) were measured as a function of the Ca^{2+} concentration. The Ca^{2+}-dependence of Trp fluorescence was indicative of a slightly higher cooperativity for Ca^{2+} binding than for Sr^{2+} binding, although asymmetry was still discernable (circles in panel A of Fig. 3), and the midpoint for pNPP hydrolysis activation (circles in panel B) was again similar to that for the rise in Trp fluorescence (1.5–2 μM). Note that, in contrast, when we measured ATP hydrolysis instead of pNPP hydrolysis, midpoints for activation by Sr^{2+} or Ca^{2+} shifted to lower metal concentrations in both cases (not shown).

4. DISCUSSION

Our simultaneous measurement of the fluorescence of both the fluorescein moiety and the Trp residues of the same FITC-labeled ATPase (Fig. 1) provides clear-cut evidence for a difference in the sensitivity to Sr^{2+} of the two different probes. When the ATPase was titrated with Sr^{2+}, the Trp fluorescence only rose for Sr^{2+} concentrations higher than those reducing the fluorescence of FITC (Fig. 2). The dependence on Sr^{2+} of the Trp signal was steeper for low than high Sr^{2+} concentrations, and matched the Sr^{2+} dependence of pNPP hydrolysis by the ATPase (Fig. 3).

How is this possible? The clue to our observations lies in the poor cooperativity with which Sr^{2+} binds to Ca^{2+}-ATPase [14–16]. The inset in Fig. 2 is a simulation of a hypothetical simple case of the sequential binding of two ions (M) with identical affinities for the ATPase, 1 and 1, in arbitrary concentration units. In this case, as pointed out by Fujimori and Jencks, the curve describing the proportion of ATPase molecules with two bound ions, $[EM_2]$, shifts to higher ion concentrations than the one describing the proportion of ATPase molecules with no bound ions ([E], or $[E_{tot}]-[E]$), and the slope of the Hill plot for the proportion of $[EM_2]$ varies from 2 to 1 when the ion concentration rises.

Independent of the precise curve shapes, our observation of a significant difference in the sensitivity to Sr^{2+} of two probes on the same ATPase molecule is therefore consistent with the presence, at equilibrium, of a significant proportion of the ATPase species with only one metal ion bound (otherwise the protein state would be either E or EM_2, with one set of spectral characteristics or the other). In other words, it fully confirms that the cooperativity for Sr^{2+} binding is poor under these conditions. In addition, the poor sensitivity to Sr^{2+} of the Trp signal, relative to that of bound FITC, and the oddly shaped curve for the Sr^{2+}-dependence of Trp fluorescence, steep at low Sr^{2+} and less steep at high Sr^{2+}, suggest that this Trp signal is mainly dominated by the proportion of ATPases with two bound metal ions, $[EM_2]$. In fact, both with respect to its midpoint and with respect to its peculiar asymmetric shape, the Sr^{2+} dependence we measured for the rise in Trp fluorescence was strikingly similar to the Sr^{2+} dependence found by Fujimori and Jencks for the proportion of ATPase reactive to [γ-^{32}P]ATP, i.e. the proportion of ATPase with

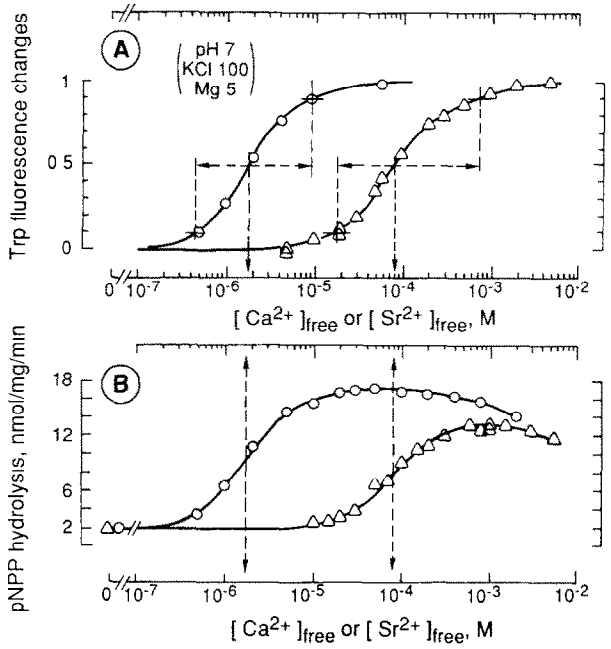

Fig. 3. Effects of Sr^{2+} and Ca^{2+} on SR Trp fluorescence and the hydrolysis rate of pNPP. Panel A: changes in Trp fluorescence. Unlabeled SR vesicles (0.1 mg/ml) were suspended in the medium described in the legend to Fig. 1. 1.2 mM EGTA (triangles) or 5 mM Mg EDTA (circles) was added first, and then various amounts of Sr^{2+} (triangles) or Ca^{2+} (circles). Fluorescence changes are plotted relative to the maximal changes, which were identical for Ca^{2+} and Sr^{2+}. Panel B: steady state rate of pNPP hydrolysis by unlabeled SR vesicles made leaky with A23187 ionophore, as a function of the free Sr^{2+} (triangles) or Ca^{2+} concentration (circles). The medium contained 100 μg/ml protein, 2 μg/ml A23187 and 3 mM pNPP (plus 1 mM extra Mg^{2+}, to keep the free Mg^{2+} concentration unaltered).

two bound Sr^{2+} ions (squares in Fig. 5 of [16]). The fact that the midpoint for activation of pNPP hydrolysis by Sr^{2+} was close to the midpoint for formation of the ATPase species with high Trp fluorescence (compare panels A and B in Fig. 3) is also consistent with the idea that the latter is dominated by the proportion of ATPase species with two bound ions. This is because during pNPP hydrolysis, pNPP binding to the ATPase species with two bound ions, or the resulting ATPase phosphorylation, is the rate-limiting step, so that the overall hydrolysis rate closely reflects the abundance of this particular species. In contrast, the rate-limiting step for hydrolysis of ATP generally follows phosphoenzyme formation, and as a result, the midpoint for the activation of ATP hydrolysis corresponds to a much lower ion concentrations than the true midpoint for ion binding in the absence of ATP (data not shown).

Within the framework of the simple sequential binding and dissociation scheme illustrated in the inset of Fig. 2, the high sensitivity of bound FITC to low Sr^{2+} concentrations (Figs. 1 and 2) suggests that the FITC fluorescence level of the ATPase species with only one Sr^{2+} ion bound, [EM], is already low, probably almost as low as the final FITC fluorescence level of the $[EM_2]$ species. This contrasts with the fluorescence of the Trp residues, which would mainly rise when the second Sr^{2+} ion binds to the ATPase. However, these tentative conclusions are only approximations, which will have to be finely retuned when the detailed mechanism of ion binding is taken into account, with its complicated sequence of binding steps and conformational changes. For instance, the drop in FITC fluorescence on formation of [EM] might result either from Sr^{2+} binding itself or from a shift in some prequilibrium preceding ion binding, as has been suggested for NBD-labeled ATPase to occur during binding of Ca^{2+} [6].

We would like to emphasize that the results collected here at 20°C and neutral pH, in the presence of 5 mM Mg^{2+} and 100 mM KCl, could be obtained because of the poor cooperativity of Sr^{2+} binding to the ATPase under these experimental conditions. This poor cooperativity rendered possible the spectroscopic differentiation of some the substeps involved in the binding of the two Sr^{2+} ions to the ATPase transport sites. Conversely, under different experimental conditions, such differentiation might turn out to be impossible, either because of different relative fluorescence levels for the various intermediates, or simply because of a higher cooperativity for ion binding. Nevertheless, the present findings open the possibility of combining equilibrium measurements such as those reported here with time-resolved measurements, which will presumably allow *kinetic* differentiation between the various ATPases species even if differentiation is not possible at equilibrium. Taking advantage of the present demonstration that Trp residues and bound FITC may differentiate between some of the substeps involved in ion binding to the ATPase, it will no doubt be interesting in the future to reinvestigate the mechanism of binding of Ca^{2+} itself to Ca^{2+}-ATPase. More generally, we would like to stress, in conclusion, the potentialities of the approach used here for the study of the many enzymes for which Trp fluorescence is an informative reporter group, and which can also be labeled by an extrinsic fluorophore with adequate spectroscopic properties.

Acknowledgments: We thank W.P. Jencks and T. Fujimori for fruitful discussions, Mathilde Dreyfus for her help in editing the manuscript, and our colleagues in Saclay for cooperative interactions!

REFERENCES

[1] Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986) Cell 44, 597–607.
[2] Inesi, G., Lewis, D., Nikko, D., Hussain, A. and Kirtley, M.E. (1992) Adv. Enzymol. 65, 183–215.
[3] Toyoshima, C., Sasabe, H. and Stokes, D.L. (1993) Nature 362, 469–471.
[4] de Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292.
[5] Stahl, N. and Jencks, W.P. (1987) Biochemistry 26, 7654–7667.
[6] Wakabayashi, S. and Shigekawa, M. (1990) Biochemistry 29, 7309–7318.

[7] Inesi, G , Kurzmack, M., Coan, C. and Lewis, D.E. (1980) J. Biol. Chem. 255, 3025–3031.
[8] Van der Kloot, W.G. and Glovsky, J. (1965) Comp. Biochem. Physiol. 15, 547–565.
[9] Weber, A., Herz, R and Reiss, I. (1966) Biochem. Z 345, 329–369.
[10] Mermier, P. and Hasselbach, W. (1976) Eur. J. Biochem. 69, 79–86.
[11] Guimaraes-Motta, H., Piedade Sande-Lemos, M. and de Meis, L. (1984) J. Biol. Chem 259, 8699–8705.
[12] Sumida, M., Hamada, M., Takenaka, H., Hirata, Y., Nishigauchi, K and Okuda, H. (1986) J. Biochem. (Tokyo) 100, 765–772.
[13] Berman, M.C. and King, S.B. (1990) Biochim. Biophys. Acta 1029, 235–240.
[14] Arav, R., Aderem, A.A and Berman, M.C. (1983) J. Biol. Chem. 258, 10433–10438.
[15] Holguin, J.A. (1986) Arch. Biochem. Biophys. 251, 9–16.
[16] Fujimori, T. and Jencks, W.P. (1992) J. Biol. Chem. 267, 18475–18487.
[17] Wakabayashi, S., Imagawa, T. and Shigekawa, M. (1990) J. Biochem. 107, 563–571.
[18] Bigelow, D J. and Inesi, G (1992) Biochim. Biophys. Acta 1113, 323–338.5
[19] Dupont, Y. (1976) Biochem. Biophys Res. Commun. 71, 544–550.
[20] Pick, U. (1981) FEBS Lett. 123, 131–136.
[21] Mitchinson, C., Wilderspin, A.F., Trinniman, B.J and Green, N.M. (1986) FEBS Lett. 146, 87–92.
[22] Champeil, P., Riollet, S., Orlowski, S., Guillain, F., Seebregts, C.J. and McIntosh, D.B. (1988) J. Biol. Chem 263, 12288–12294.
[23] Orlowski, S. and Champeil, P (1991) Biochemistry 30, 352–361.
[24] König, K. G and Hasselbach, W. (1984) Z. Naturforsch. 39c, 282–284.
[25] Martell, A.E. and Smith, R.M. (1974) Critical Stability Constants, vol. 1 and vol. 3, Plenum Press, New York.
[26] Tsien, R. and Pozzan, T. (1989) Methods Enzymol. 172, 230–262.
[27] Combettes, L., Claret, M. and Champeil, P (1993) Cell Calcium, 14, 279–292