Inhibition of the skeletal muscle ryanodine receptor calcium release channel by nitric oxide

László G. Mészáros*, Igor Minarovic, Alexandra Zahradnikova**

Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA 30912, USA

Received 27 September 1995; revised version received 15 December 1995

Abstract NO donors were found to reduce the rate of Ca^{2+} release from isolated skeletal muscle sarcoplasmic reticulum (SR) and the open probability of single ryanodine receptor Ca^{2+} release channels (RyRCs) in planar lipid bilayers, and these effects were prevented by the NO quencher hemoglobin and reversed by 2-mercaptoethanol. Ca^{2+} release assessed in skeletal muscle homogenates was also reduced by NO that was generated in situ from L-arginine by endogenous, nitro-L-arginine methylester-sensitive NO-synthase. The effect of NO on the RyRC might explain NO-induced depression of contractile force in striated muscles and, since both RyRC isoforms and NOS isoenzymes are ubiquitous, may represent a wide-spread feedback mechanism in Ca^{2+} signaling; i.e. Ca-dependent activation of NO production and NO-evoked reduction of Ca^{2+} release from intracellular Ca^{2+} stores.

Key words: Ryanodine receptor; Nitric oxide; Calcium release; Sarcoplasmic reticulum

1. Introduction

Nitric oxide (NO) as a biological messenger molecule has been assigned to a number of cellular functions in a wide variety of tissues [1,2], ranging from the regulation of the vascular tone to neuronal plasticity. More recently, NO has been implicated in cytokine- and endotoxin-evoked decreases in cardiac contractility [3,4] and found to depress contractile function in fast skeletal muscle where the brain type constitutive NO-synthase (NOS) has been shown to be expressed [5]. The reduction of skeletal muscle contractile force by NO was only partially ascribable to its stimulatory effect on guanylate cyclase [5], the often reported signaling pathway in NO action [1,2,6]. Since in striated muscles there is a direct relationship between the extent of contraction and the amount of Ca^{2+} released from the sarcoplasmic reticulum (SR) through the ryanodine receptor Ca^{2+} release channel (RyRC), we have asked the question whether the Ca^{2+} release machinery, i.e. the RyRC, is directly targeted by NO.

Here we report evidence that identifies the Ca^{2+} release mechanism, most likely the RyRC itself, as an NO target in skeletal muscle: we show that NO generated either by exogenous NO donors or enzymatically from L-arginine in the NO-synthase reaction in situ decreases Ca^{2+} release activity from SR, which parallels an NO-induced reduction in the open probability of single RyRCs fused into planar lipid bilayers.

*Corresponding author. Fax: (1) (706) 721-3168.

**On leave from the Institute of Molecular Physiology and Genetics, Slovak Academy of Science, Bratislava, Slovakia.

2. Materials and methods

2.1. Preparations

Heavy sarcoplasmic reticulum (SR) vesicles were prepared from fast twitch muscles of rabbit hind legs by differential [10] and sucrose gradient centrifugation [11]. The vesicles isolated by using the differential centrifugation protocol were used in the flux, while the gradient-purified preparations were used in the single channel studies. The final pellets were resuspended in a solution of 75 mM KCl, 150 mM sucrose, 20 mM MOPS, pH 6.8, also containing a mixture of protease inhibitors [12] and stored at −80°C until their use. Skeletal muscle homogenate (i.e. a mitochondria-free membrane plus cytosol fraction) was prepared by an overnight dialysis against 500 volume of the above KCl-sucrose solution of the supernatant obtained in the first step of the above differential centrifugation protocol.

2.2. Ca^{2+} flux measurements

Ca^{2+} uptake and release were followed by monitoring the absorbance changes of arsenazo III at 650–680 nm using a beam splitter with interference filters and a 600 nm cut-on filter in the incoming light path as described previously [13 and ref. 17 therein]. The photomultiplier signals were amplified with an On-Line Instruments dual channel amplifier which was interfaced through an A/D board with a 386 computer for data acquisition. Ca^{2+} uptake was initiated by the addition of 8 mM acetylphosphate (AcP) to 50–80 μg/ml SR protein suspended in 150 mM KCl, 20 mM (3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 6.8, 0.5 mM $MgCl_2$ and 10 μM arsenazo III (KCl-MOPS medium). After the completion of Ca^{2+} uptake, Ca^{2+} release was induced by hand-mixing or, to avoid optical artifacts, rapid mixing of caffeine. In Ca^{2+} release experiments carried out with calmodulin-supplemented skeletal muscle homogenates, trifluoperazine (TFP) was added together with caffeine to prevent calmodulin from influencing the release channel [14]. The arsenazo III signal was calibrated to total Ca^{2+} concentrations in the medium and the non-linear portion of the signals were corrected on-line using the Labtech Notebook data-acquisition system.

2.3. Single channel measurements

Bilayers consisting of 7:3 phosphatidyl ethanolamine/phosphatidyl choline (Avanti Polar Lipids, 50 mg/ml in decane) were formed across a 150 μm aperture in a Teflon chamber. The current measurements and data acquisition were performed as described previously [15], except that unitary currents were measured with a BLM-120 bilayer amplifier (Bio-Logic, France), the leak and capacitance components were electronically subtracted and the data were low-pass filtered at 2 kHz using a home-made filter device. $CsCH_3SO_3$ solutions (i.e. Cs^+ as current carrier) buffered to pH 7.4 with 10 mM MOPS (250 mM *cis*, 50 mM *trans*) were used to isolate the channel from other ionic conductances and to improve signal to noise ratio as described [16]. Single channel conductance was determined by measuring the current amplitudes at 0, +20 and +30 mV holding potential of fully resolved openings. The analysis of single channel records was carried out on a 386 computer, using the FETCHAN (Axon Instr.) and TRANSIT (Baylor College of Medicine, Houston, TX) softwares.

3. Results and discussion

The time course of acetylphosphate (AcP)-supported Ca^{2+} uptake into heavy SR vesicles derived from terminal cisternae [17] is shown in Fig. 1A (control, trace a). The inclusion of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) in the me-

SSDI 0014-5793(96)00003-8

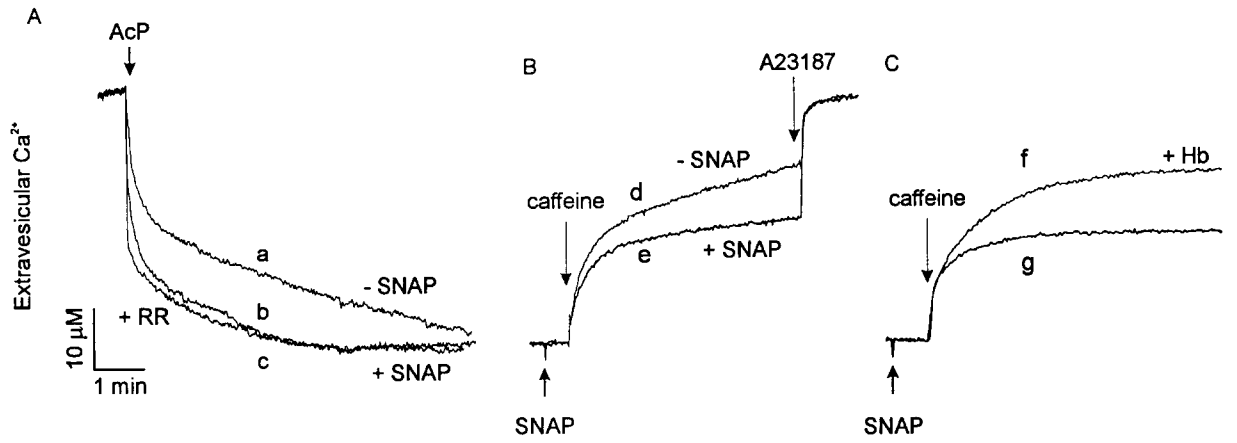

Fig. 1. The effects of SNAP on Ca^{2+} uptake and release in isolated SR vesicles. Ca^{2+} uptake (traces a–c) initiated by acetylphosphate addition (AcP, 20 mM) and release induced by the addition of 6.6 mM caffeine after completion of Ca^{2+} uptake (traces d–g) were followed spectrophotometrically (see section 2). SNAP (10 μM) was either added prior to AcP (traces b,c) or where indicated (traces e–g). Ruthenium red (5 μM) was present in trace c, oxygenated hemoglobin (Hb, 20 μM) in trace f. Ca-ionophore A23187 (2 μM) was added where indicated. Representative traces are shown.

dium (trace b) resulted in an apparent increase in the rate of the second slow phase of Ca^{2+} accumulation, without having any appreciable effect on its initial rate. This suggests that SNAP, instead of directly influencing the SR Ca^{2+} pump, was most likely to reduce the rate of Ca^{2+} release. That this was indeed the case is indicated by the findings that in the presence of the Ca^{2+} release blocker ruthenium red [10,17] the rates of Ca^{2+} uptake with and without SNAP were indistinguishable (not shown) and that, as illustrated in Fig. 1B, the rate of Ca^{2+} release induced by caffeine was significantly lowered in the presence of SNAP (trace e, as compared to the control trace d). The addition of the NO-quencher hemoglobin to the medium prior to SNAP addition (trace f) prevented the NO donor from decreasing SR Ca^{2+} release activity, indicating that the

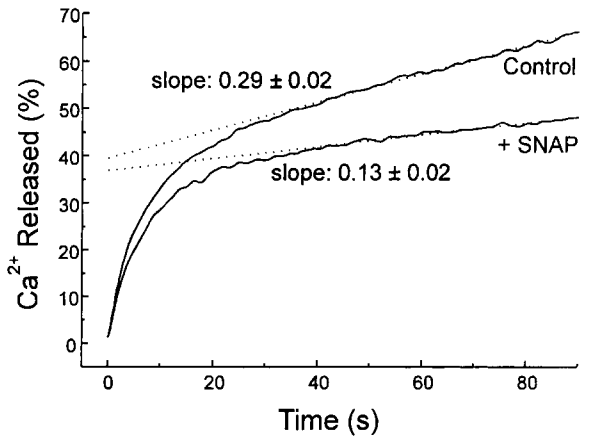

Fig. 2. The kinetics of SNAP-inhibition of Ca^{2+} release. The measurements were carried out as described in the section 2. Caffeine (6.6 mM final concentration) was rapidly mixed to SR vesicles that were preloaded with Ca^{2+} as shown in Fig. 1. The released (total) Ca^{2+} is expressed as the percentage of the total luminal Ca^{2+} that is releasable upon A23187 addition (see also Fig. 1B). The traces are the averages of 22 and 16 individual sweeps of control experiments and those with SNAP (10 μM), respectively, and were collected from experiments with 2 separate SR preparations. Dotted lines are the best fits to the linear portion of the time course and the computed slopes are shown. Initial rates were (as computed by fitting of exponentials; not shown): 7.51 and 5.83 (%/s) for control and SNAP, respectively.

principal agent responsible for release inhibition was NO liberating from SNAP. This is also supported by the findings that other NO donors with rather different chemical structure (3-morpholino-sydnonimine, SIN-1 and sodium nitroprusside, SNP) were also found to inhibit SR Ca^{2+} release (not shown).

Fig. 2 depicts averaged traces of caffeine-induced Ca^{2+} release in the absence and presence of SNAP. These traces were recorded as those in Fig. 1, except that a Durrum two-syringe rapid mixer was used to avoid optical artifacts due to mixing. SNAP only caused a slight reduction in the initial rate (22%), but reduced the rate in the second slow phase of Ca^{2+} release by about 65% (see the dotted lines in Fig. 2 that are best fits to the linear portion of the time courses). The same type of kinetic effects, i.e. slight reduction of the initial, but marked decrease in the second phase, were observed with SIN-1 and SNP as NO donors (not shown). The analysis of the release time courses (to be published separately) with and without SNAP suggests that the mechanism of NO-inhibition is the promotion of channel inactivation, rather than the blockage of the release channel.

Kobzik et al. [5] found that the Ca^{2+}/calmodulin-dependent constitutive brain isoform of nitric oxide synthase (NOS) is expressed in fast skeletal muscle. This has provided a possibility

Table 1
Effect of SNAP on single RyRC behavior and its removal by 2-mercaptoethanol

Parameter	Control	SNAP	SNAP+1.4 mM 2-mercaptoethanol
Open probability* (%)	10.43 ± 0.82	4.1 ± 0.59	12.31 ± 4.13
Average open time** (ms)	1.21 ± 0.16	0.85 ± 0.07	1.28 ± 0.02
Average number of openings/ segment***	30.9 ± 1.3	16.57 ± 2.2	46.0 ± 12.8

t-test: for SNAP vs. Control: *$P < 0.001$; **$P < 0.1$; ***$P < 0.01$ (n, i.e. number of independent experiments, =4); for SNAP + 2-mercaptoethanol vs. Control: *-**-*** - n.s. ($n = 4$). Total of 484 segments for control, 974 for SNAP and 243 for SNAP+2 mercaptoethanol. Each segment was 409.6 ms long. The effects of SNAP developed within few seconds after addition.

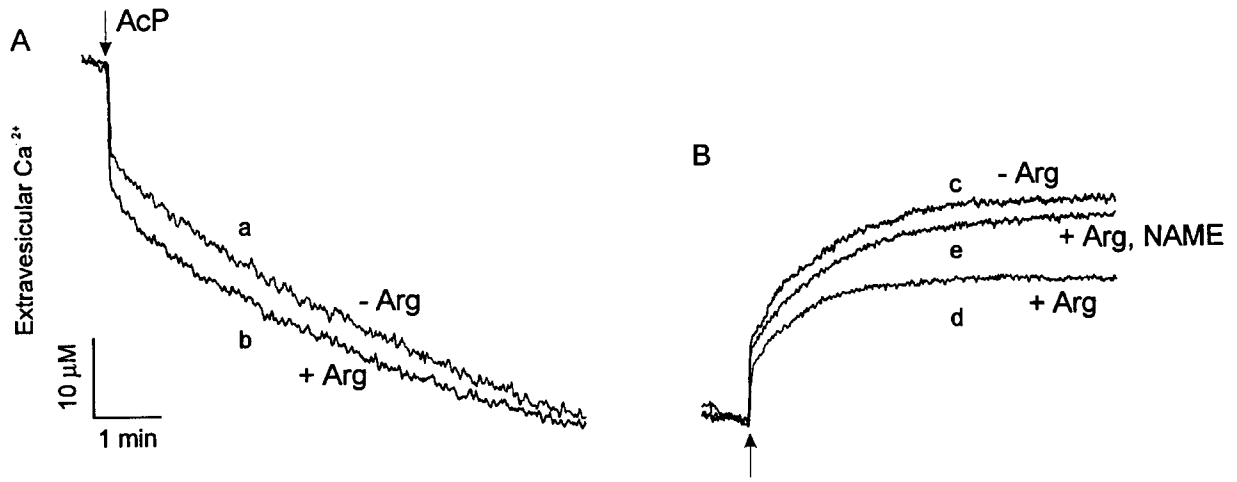

Fig. 3. The effects of NO generated by endogenous NOS on Ca^{2+} uptake and release in skeletal muscle homogenates. AcP (at the arrow) was added to 5-fold diluted (0.8–1.2 mg/ml) homogenate in KCl-MOPS medium supplemented with 10 µg/ml calmodulin, 5 µM FAD, 5 µM FMN, 10 µM tetrahydro-L-biopterin, 0.1 mM NADPH (tracec, marked as −Arg) plus 0.5 mM L-arginine (trace b, d and e, marked as +Arg). After completion of Ca^{2+} uptake, caffeine (6.6 mM) and TFP (400 µM) were added (at the arrow). In control experiments with TFP present, the Ca^{2+} release flux was completely blocked by 5 µM ruthenium red (not shown). L-NAME (2.5 mM) was added before Ca^{2+} uptake was initiated (trace e, marked as +Arg, NAME). Representative traces are shown.

to test whether SR Ca^{2+} release is also responsive to endogenously generated NO. Thus, we studied SR Ca^{2+} uptake (Fig. 3A) and release (Fig. 3B) in skeletal muscle homogenates supplemented with NOS substrates (L-arginine, NADPH), cofactors (FMN, FAD, tetrahydro-L-biopterin) and calmodulin [18]. When L-arginine was omitted from the medium, Ca^{2+} uptake was found to be slower (trace a as compared to trace b), while Ca^{2+} release induced by caffeine (plus TFP, see section 2) was significantly faster (trace c as compared to trace d). Furthermore, the addition of the NOS inhibitor nitro-L-arginine methylester (L-NAME) was apparently able to antagonize the effect of L-arginine (trace e vs. trace d), i.e. increased the release rate close to control (-L-arginine) levels. These results indicate that NO generated in situ from L-arginine in a L-NAME-sensitive NOS reaction was also capable of reducing Ca^{2+} release rate.

The above results obtained from flux measurements suggest that NO might inhibit the RyRC, the principal Ca^{2+} release pathway in striated muscles. To directly test this possibility, sucrose gradient purified SR vesicles were fused into planar

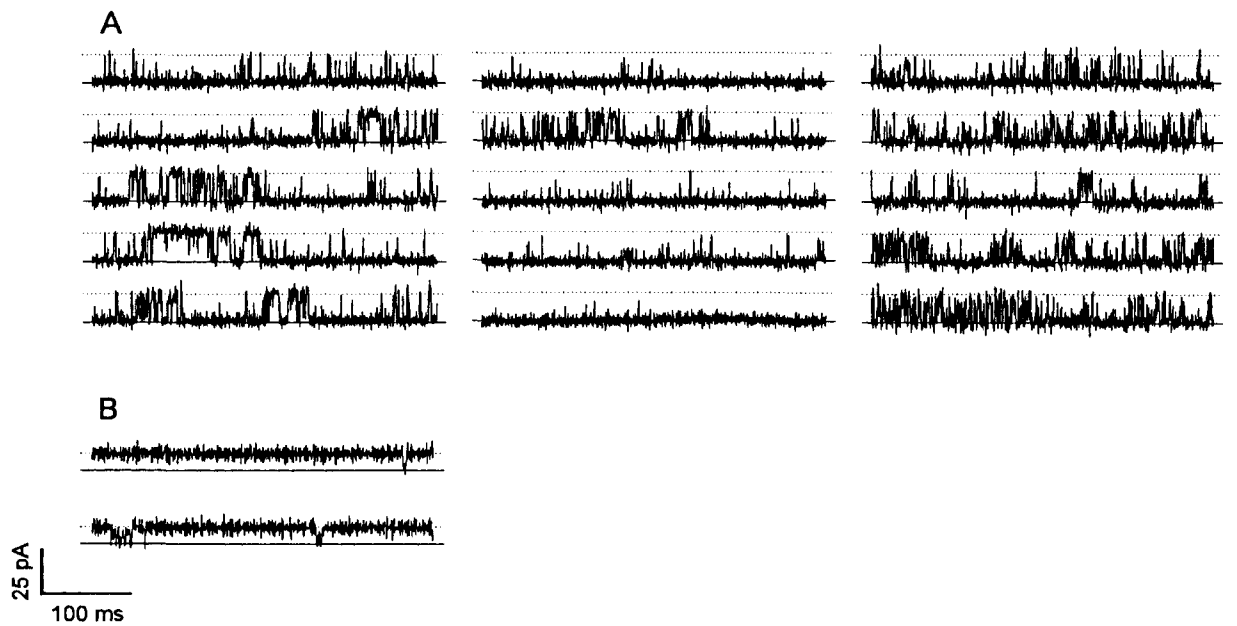

Fig. 4. Inactivation of a single RyRCs by SNAP and its reversal by 2-mercaptoethanol (A). The channel was activated by 20 µM Ca^{2+} and single channel currents were recorded at +30 mV (left), then 0.1 mM SNAP was added (middle) which was followed by the addition of 1.4 mM 2-mercaptoethanol in the continuing presence of SNAP (right). B: The effect of ryanodine. Control channel currents shown in A had an amplitude of 19.5 pA and short mean open time. About 30 s after the addition of 10 µM ryanodine, single channel currents abruptly changed to ones with amplitude of 8.9 pA and long open time (18).

lipid bilayers [15,19] and unitary Cs^+ currents of single RyRCs [16] were studied in the presence and the absence of NO donors. The single channels in the bilayer were identified by both their conductance (408 ± 12 pS) and their typical response [19] to ruthenium red (not shown) as well as to ryanodine (Fig. 4B). Fig. 4A illustrates a set of consecutive episodes in representative channel records before and after the addition of SNAP (to the *cis* chamber) and after a subsequent addition of 2-mercaptoethanol. As seen from the records and the data in Table 1, which summarizes the averages of the channel parameters measured in 4 separate experiments, SNAP caused a significant decrease in the number of openings which resulted in a significant (about 60%) decrease in the overall open probability. (Note that the mean open time was not significantly changed by SNAP.) Mercaptoethanol added after SNAP apparently resulted in a recovery of both the number of openings and open probabilities to control levels (Table 1) and, when added before SNAP (not shown), it prevented the NO donor-induced decrease in open probability without having any appraciable effect on the control. This strongly suggests that the probable mechanism of NO action is the modification of highly reactive cysteine residues of RyRC [9], which are known to alter the kinetics properties of skeletal SR Ca^{2+} release [8,9]. Similar single channel results were obtained with another NO donor, SIN-1 (not shown).

In summary, NO donor compounds that are known to generate NO in aqueous solutions inhibit Ca^{2+} release from isolated SR vesicles in an NO-quencher (hemoglobin) preventable fashion. Furthermore, in skeletal muscle homogenates, Ca^{2+} release activity is decreased when the medium was supplemented with L-arginine (in the presence of other substrates and cofactors of the Ca^{2+}/calmodulin-dependent NOS). This effect of L-arginine was prevented by L-NAME, the inhibitor of NOS. These together indicate that NO inhibits SR Ca^{2+} release. Since the NO donor SNAP was also found to decrease the open probability of single RyRCs incorporated into planar lipid bilayers, the effect of NO on SR Ca^{2+} release is most likely due to its influence on the RyRC itself. Kinetic considerations suggest that the mechanism of NO action on the release channel is promoting channel inactivation.

In control experiments (not shown), we could rule out the possibility that an NO-activated cGMP signaling pathway would be responsible for the NO action we describe here, since: (i) exogenously added cGMP (up to 50 μM) induced no inhibition of SR Ca^{2+} release (in either isolated SR vesicles or in homogenates) and (ii) radio-immune-assay for cGMP showed no evidence for the presence of GTP contamination in either preparations (note that the homogenate preparations were dialyzed before use). Therefore, we conclude that NO directly affects the SR Ca^{2+} release machinery, i.e. the RyRC.

The effect of NO on SR Ca^{2+} release could account for the previously observed NO-induced force reduction in both skeletal [5] and cardiac muscle [3,4]. Whether or not NO has a role in regulating muscle contraction on a twitch-by-twitch (or beat-by-beat) basis is too early to speculate. It is worthwhile to note, however, that, since the occurrence of both the RyRCs [20] and the constitutive form of NOS [1,2] appears to be ubiquitous, the influence of NO on the release channel together with the known Ca-requirement of NO-generation via the Ca^{2+}/calmodulin-dependent isoform of NOS [1,2,18] might represent an important regulatory feedback loop, in which Ca^{2+} activates the production of NO that, in return, controls the extent of the RyRC-mediated Ca^{2+} rise in the cell. In this respect, it will be important to test how the inositol 1,4,5-trisphosphate-controlled Ca^{2+} release process, which is also known to be responsive to -SH reagents [21], would be influenced by NO. The possible operation of the above feedback mechanism is especially intriguing, when a seemingly antagonistic relationship between the stimulant Ca^{2+} and the tranquilizer NO is considered in the light of the proposed retrograde messenger role of NO in neurons [22,23] and its muscle relaxant effect in smooth [1,2] and striated muscles [3–5].

References

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–142.
[2] Nathan, C. (1992) FASEB J. 6, 3051–3063.
[3] Finkel, M.S., Oddis, C.V., Jacob, T.D., Watkins, S.C., Hattler, B.G. and Simmons, R.L. (1992) Science 257, 387–389.
[4] Brady, A.J.B., Poole-Wilson, P.A., Harding, S.E. and Warren, J.B. (1992) Am. J. Physiol. 263, H1963–1969.
[5] Kobzik, L., Reid, M.B., Bredt, D.S. and Stamler, J.S. (1994) Nature 372, 546–549.
[6] Schmidt, H.H.H.W., Lohmann, S.M. and Walter, U. (1993) Biochim. Biophys. Acta 1178, 153–175.
[7] Butler, A.R., Flitney, F.W. and Williams, D.L.H. (1995) Trends Pharmacol. Sci. 16, 18–22.
[8] Abramson, J.J. and Salama, G. (1989) J. Bioenerg. Biomembr. 21, 283–294.
[9] Liu, G., Abramson, J.J., Zable, A.C. and Pessah, I.N. (1994) Mol. Pharmacol. 45, 189–194.
[10] Ohnishi, S.T. (1979) J. Biochem. 86, 1147–1157.
[11] Meissner, G. (1984) J. Biol. Chem. 259, 2365–2371.
[12] Ikemoto, N., Ronjat, M., Mészáros, L.G. and Koshita, M. et al. (1989) Biochemistry 28, 6764–6771.
[13] Mészáros, L.G. and Ikemoto, N. (1985) J. Biol. Chem. 260, 16076–16079.
[14] Smith, J.S., Rousseau, E. and Meissner, G. (1989) Circ. Res. 64, 352–359.
[15] Zahradnikova, A. and Palade, P. (1993) Biophys. J. 64, 991–994.
[16] Fill, M., Coronado, R., Mickelson, J.R., Vilven, J., Ma, J., Jacobson, B.A. and Louis, C.F. (1990) Biophys. J. 57, 471–476.
[17] Chu, A., Volpe, P., Costello, B. and Fleischer, S. (1986) Biochemistry 25, 8315–8319.
[18] Klatt, P., Heinzel, B., John, M., Kastner, M., Böhme, E. and Mayer, B. (1992) J. Biol. Chem. 267, 11374–11378.
[19] Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y. and Meissner, G. (1988) Nature 331, 315–319.
[20] Sorrentino, V. and Volpe, P. (1993) Trends Pharmacol. Sci. 14, 98–102.
[21] Hilly, M., Pietri-Rouxel, F., Coquil, J.F., Guy, M. and Mauger, J.P. (1993) J. Biol. Chem. 268, 16488–16493.
[22] Knowles, R.G., Palacios, M., Palmer, R.M.J. and Moncada, S. (1989) Proc. Natl. Acad. Sci. USA 86, 5159–5162.
[23] Wang, T., Xie, Z. and Lu, B. (1995) Nature 374, 262–266.