Volume 314, number 1, 81–84 FEBS 11833 December 1992


# BisG10, a $K^+$ channel blocker, affects the calcium release channel from skeletal muscle sarcoplasmic reticulum

Bruno Allard[a], Marie-Jo Moutin[b] and Michel Ronjat[b]

[a]*Laboratoire de Physiologie des Eléments Excitables, URA 180 CNRS, 43 bd du 11 novembre 1918, 69622 Villeurbanne Cedex, France* and [b]*Laboratoire de Biophysique Moléculaire et Cellulaire, URA 520 CNRS, CENG, BP 85X, 38041 Grenoble, France*

Received 5 October 1992; revised version received 19 October 1992

The action of bisG10, a potent $K^+$ channel inhibitor, was tested on the $Ca^{2+}$ release from isolated sarcoplasmic reticulum vesicles of rabbit skeletal muscle. Using a rapid filtration technique, we found that the drug inhibited $Ca^{2+}$-induced $Ca^{2+}$ release elicited in the presence of extravesicular $K^+$ as counter-ion. This inhibition was not reversed by the addition of valinomycin and still occurred when $Cl^-$ was used as co-ion, indicating that not only $K^+$ channels are involved in the inhibiting effect. We found that bisG10 decreased the binding of ryanodine to sarcoplasmic reticulum vesicles, showing that bisG10 is able to block the sarcoplasmic reticulum $Ca^{2+}$ release channel.

Sarcoplasmic reticulum; Ryanodine receptor; Calcium release; BisG10

## 1. INTRODUCTION

The muscle sarcoplasmic reticulum (SR) is an intracellular membrane compartment specialized for the cyclical uptake, storage and release of $Ca^{2+}$ ions. The $Ca^{2+}$ release from SR is one of the key steps in excitation–contraction coupling. During the last few years different groups have described the characteristics of this release and shown that ryanodine, a plant alkaloid, does affect the $Ca^{2+}$ release from SR. The ryanodine receptor has then been identified, purified, sequenced and shown to possess $Ca^{2+}$ channel properties similar to those of the $Ca^{2+}$ channel involved in the $Ca^{2+}$ release from SR [1,2].

In parallel to the study of $Ca^{2+}$ release, a number of laboratories with different experimental approaches including light scattering, isotope flux measurements and fusion of SR vesicles into planar bilayers, have given evidence indicating that the SR membrane of skeletal muscle is selectively permeable to small monovalent cations [3–5]. Since, a channel that displays a high conductance for $K^+$ in the SR membrane has been described and the electrical properties of this channel well defined [5–7]. The physiological role of this $K^+$ permeability is still an important question. Because of the elevated cytoplasmic $K^+$ concentration and the high conductance of the SR $K^+$ channel, it has been suggested that this channel may provide a mechanism for allowing $K^+$ movement to compensate the charge imbalance produced by $Ca^{2+}$ fluxes across the SR membrane during the contraction/relaxation cycles [8]. Several studies support the hypothesis that $K^+$ ions are the counter-ions for $Ca^{2+}$ fluxes. (i) Electron probe analysis showed that $Ca^{2+}$ release from the SR during a tetanus was accompanied by an increase in the concentration of $K^+$ ions in the terminal cisternae [9]. (ii) Rapid filtration studies on isolated SR vesicles indicated that the $Ca^{2+}$-induced $Ca^{2+}$ release was dependent upon the presence of $K^+$ in the release medium and influenced by the $K^+$ gradient created across the SR membrane [10]. (iii) In skinned muscle fiber, substitution of the impermeant ion choline for $K^+$ caused a decrease in the caffeine-induced $Ca^{2+}$ release rate [11].

One way to demonstrate the involvement of the SR $K^+$ channels in counter-ion movement during $Ca^{2+}$ fluxes has been to study the effect of $K^+$ channel blockers. Using skinned musle fibers, Fink and Stephenson [12] showed that the ability of SR to load $Ca^{2+}$ was significantly increased by the presence of TEA, 4-AP, decamethonium or procaine, whose efficiency on the SR $K^+$ channel has been demonstrated in planar bilayer [13,14]. On the same preparation, Abramcheck and Best [11] indicated that bisG10, one of the most effective SR $K^+$ channel blockers [15], caused a concentration-dependent decrease in the caffeine-induced $Ca^{2+}$ release rate. In both studies the target of these pharmacological compounds was postulated to be the SR $K^+$ channel.

In this paper, we investigate the effects of bisG10 on the $Ca^{2+}$ release from isolated SR vesicles. We show that

*Correspondence address:* B. Allard, Laboratoire de Physiologie des Eléments Excitables, URA 180 CNRS, 43 bd du 11 novembre 1918 Bat 401B, 69622 Villeurbanne Cedex, France. Fax: (33) (78) 94 95 85.

*Abbreviations:* bisG10, 1,10-bis-guanidino-*n*-decane; SR, sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BTP, bis-tris propane; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N,N'*-tetraacetic acid; HEPES, (*N*-[2-hydroxy-ethyl]piperazine-*N'*-[2-ethanesulfonic] acid.

bisG10 is a potent blocker of the $Ca^{2+}$-induced $Ca^{2+}$ release but that besides its effect on the $K^+$ channel, it does interact directly with the ryanodine receptor. In view of these results, the correlation between the inhibition of the $K^+$ channel activity and the inhibition of the $Ca^{2+}$ release function has to be reconsidered since it was mainly based on the specificity of effect of bisG10 on the $K^+$ channel.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of sarcoplasmic reticulum vesicles*

SR vesicles were prepared from rabbit skeletal muscle by the technique of Hasselbach and Makinose [16] as modified by Dupont [17]. The vesicles (about 10–20 mg of protein /ml) were suspended in solutions containing buffers (MOPS/BTP) and saccharose. Samples (0.5 or 1 ml) were rapidly frozen in liquid nitrogen where they were stored until used.

### 2.2. *Rapid filtration measurements of $Ca^{2+}$ release*

#### 2.2.1. Solutions

For experiments with extravesicular $K^+$ as counterions, vesicles (3.6 mg/ml) were passively loaded by incubating them for 1 h at room temperature in solution containing 180 mM MOPS, 30 mM BTP, 5 mM $^{45}$Ca-methane sulfonate (CaMes) (4 $\mu$Ci/ml) at pH 6.8. Release solution contained 50 mM KOH, 180 mM MOPS, 2 mM EGTA, 1.5 mM CaMes (1 $\mu$M free $Ca^{2+}$), 5 mM ATP at pH 6.8. For experiments with intravesicular $Cl^-$ as co-ions, the loading solution contained 5 mM $^{45}$Ca-Mes, 50 mM HCl and 30 mM BTP at pH 6.8. Release solution contained 180 mM MOPS, 30 mM BTP, 2 mM EGTA, 1.5 mM Ca-Mes (1 $\mu$M free $Ca^{2+}$), 5 mM ATP at pH 6.8. The osmotic conditions were fixed around 250 mOsm during the loading using sucrose and were conserved during the filtration. Free $Ca^{2+}$ concentrations of the various solutions were established using EGTA and calculated with the dissociation constants given by Vianna [18]. The contaminating $Ca^{2+}$ was supposed to be no more than 10 $\mu$M.

#### 2.2.2. Measurement of $Ca^{2+}$ release by the rapid filtration technique

$Ca^{2+}$ release was measured using the Bio-Logic rapid filtration system (Grenoble, France). The loaded vesicles were diluted 40-fold into a solution containing the same ionic composition as the loading solution but in the absence of $Ca^{2+}$ and with 20 mM EGTA. Then, 1.9 ml of the diluted vesicles (about 0.2 mg of protein) were applied to a Millipore filter (0.65 $\mu$M). The loaded filters were prewashed with 5 ml of a solution containing the same ionic composition as the loading solution but in the absence of $Ca^{2+}$ and with 2 mM EGTA to eleminate the non-specific $^{45}Ca^{2+}$ binding. At 30 s following the vesicles dilution, the release solution was rapidly passed through the filters using the Rapid Filtration apparatus until the desired release time was reached. Millipore filters were then dissolved in 7 ml liquid scintillation cocktail Ready Safe, Beckman, and the radioactivity was measured in a SL 3000 scintillation counter.

### 2.3. *[$^3$H]ryanodine binding experiments*

SR vesicles were incubated at 37°C in a medium containing 0.5 M NaCl, 15 mM HEPES, 2 mM $CaCl_2$ and 2 mM EGTA (free concentration of $Ca^{2+}$ = 30 $\mu$M) with 10 nM [$^3$H]ryanodine (NEN) or 10 nM [$^3$H]ryanodine + 90 nM cold ryanodine. At the desired time, 500 $\mu$l of the incubated vesicles were applied to a Whatman GF/B filter. The loaded filters were washed with 15 ml of a solution containing 0.5 M NaCl and 15 mM HEPES in order to eliminate the non-specific [$^3$H]ryanodine binding. The filters were then dissolved in 7 ml liquid scintillation and the radioactivity was measured.

### 2.4. *Synthesis of bisG10*

The compound bisG10 (1,10-bis-guanidino-*n*-decane) was synthesized according to Garcia and Miller [15]. The product obtained was the 1,10-bis-guanidino-*n*-decane · $H_2SO_4$.

## 3. RESULTS

We have first investigated the effect of bisG10 on the $Ca^{2+}$ release from isolated SR vesicles using a rapid filtration technique (Fig. 1). $Ca^{2+}$ release was induced by 1 $\mu$M $Ca^{2+}$ and 5 mM caffeine in the presence of 5 mM ATP at pH 6.8 with 50 mM $K^+$ in the release medium. Under these conditions about 50% of the passively loaded $Ca^{2+}$ was rapidly released within 500 ms in control. The apparent rate constant of $Ca^{2+}$ release was 5.7 $s^{-1}$. According to Garcia and Miller [15], for bisG10 to inhibit the monovalent cation fluxes, it must be preincubated for several minutes with the SR vesicles. Thus, in our experiments, bisG10 was added in the incubation medium 10 minutes prior to the release procedure. The drug was also put in the release solution. The results presented on Fig. 1 show that bisG10 inhibits the $Ca^{2+}$ release in a dose-dependent manner. Rate constant of release was reduced to 40% of the control value in the presence of 70 $\mu$M and calcium release was completely abolished in the presence of 360 $\mu$M bisG10.

Since bisG10 was previously described as a potent inhibitor of the SR $K^+$ channel, we investigated the putative reversion of its effect on $Ca^{2+}$ release by the $K^+$ ionophore valinomycin [19]. If bisG10 actually blocks the SR $K^+$ channel, valinomycin, by reestablishing SR $K^+$ permeability, might reverse the inhibitory effect of bisG10. Valinomycin (10 $\mu$M) was added directly in the incubation medium at the beginning of the loading and in the release medium. As already mentioned by Moutin and Dupont [10], valinomycin did not significantly modify the $Ca^{2+}$ release rate under control conditions (not shown). Fig. 2A shows that the $Ca^{2+}$ release inhibition observed in the presence of bisG10 (70 $\mu$M) was not reversed in the presence of valinomycin. This was true for all the bisG10 concentrations tested (70 $\mu$M, 210 $\mu$M, 360 $\mu$M) (not shown). Similar results were obtained using the other monovalent ionophore gramicidin (10

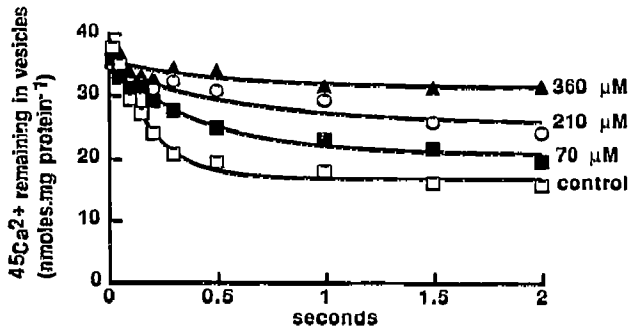


Fig. 1. Effects of increasing concentrations of bisG10 on the $Ca^{2+}$ release induced by 1 $\mu$M $Ca^{2+}$ and 5 mM caffeine, with extravesicular $K^+$ (50 mM) as counter-ion.

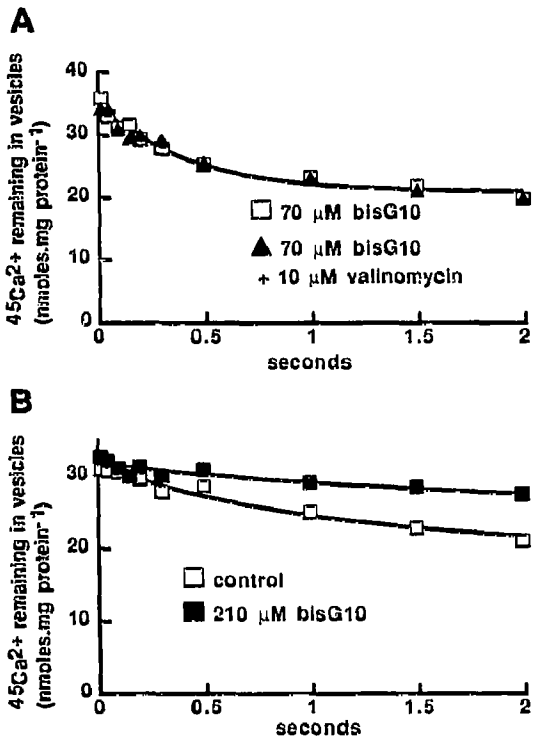


Fig. 2. (A) Effect of 10 $\mu$M valinomycin on the $Ca^{2+}$ release induced by 1 $\mu$M $Ca^{2+}$ and 5 mM caffeine, in the presence of bisG10 (70 $\mu$M) with extravesicular $K^+$ (50 mM) as counter-ion. (B) Effect of bisG10 (210 $\mu$M) on the $Ca^{2+}$ release induced by 1 $\mu$M external $Ca^{2+}$ and 5 mM caffeine with intravesicular $Cl^-$ (50 mM) as co-ion.

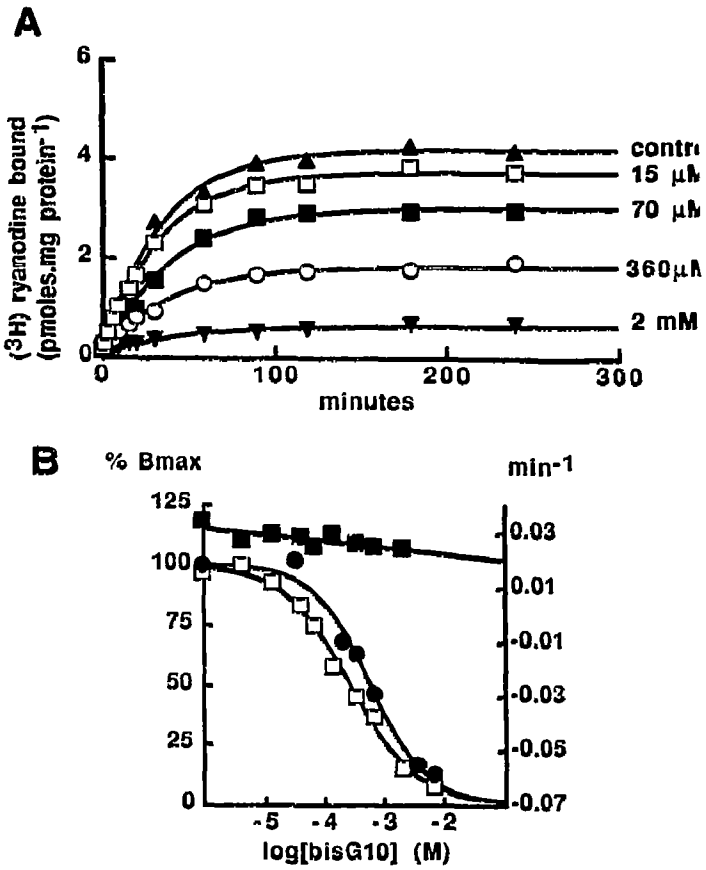


Fig. 3. (A) Inhibition of [$^3$H]ryanodine (10 nM) binding to SR vesicles ($B_{max}$) by increasing concentrations of bisG10. (B) Concentration dependence of the inhibition of the ryanodine binding to SR vesicles by bisG10, in the presence of 10 nM ryanodine (□), and 100 nM ryanodine (●). Absence of effect of bisG10 on the kinetic constants of the binding (■).

$\mu$M). This result seems to indicate that the inhibition of $Ca^{2+}$ release by bisG10 is not only due to an effect on the $K^+$ channel of the SR membrane.

To further test this hypothesis, we performed $Ca^{2+}$ release experiments with $Cl^-$ as co-ion for which the SR membrane is known to be highly permeable [20]. In these experiments, SR vesicles were incubated in the presence of 50 mM $Cl^-$ in the loading solution. As previously described by Moutin and Dupont [10], the rate constant of $Ca^{2+}$ release measured in the presence of intravesicular $Cl^-$ was considerably smaller (0.9 $s^{-1}$) than those measured in the presence of extravesicular $K^+$ (Fig. 2B). Fig. 2B shows that, under these conditions, i.e. when the intravesicular charge deficit induced by $Ca^{2+}$ release was compensated by an anionic efflux, the rate constant of $Ca^{2+}$ release was still reduced from 0.9 $s^{-1}$ in control to 0.47 $s^{-1}$ by addition of bisG10 (210 $\mu$M). Thus, under conditions where $K^+$ channels are not involved in $Ca^{2+}$ release, bisG10 still exerts its inhibiting effect on the $Ca^{2+}$ release. These findings also suggest that bisG10 acts on other sites besides the $K^+$ channel.

A likely hypothesis could be that the target of bisG10 is the $Ca^{2+}$ release channel itself. This was tested by studying the effect of bisG10 on the radiolabelled ryanodine binding capacity of the SR vesicles, which constitutes a usefull tool to investigate the SR $Ca^{2+}$ channel activity.

SR vesicles were incubated at 37°C with 10 nM [$^3$H]ryanodine in the presence of 30 $\mu$M $Ca^{2+}$. Under these conditions, SR vesicles maximally bound 3.9 pmol ryanodine/mg protein with a rate constant of 0.03 $min^{-1}$ (Fig. 3A). When bisG10 was added in the incubation medium, the maximal amount of bound ryanodine was reduced in a dose-dependent manner (Fig. 3A). The concentration of bound ryanodine decreased from 3.9 in control conditions to 3.7, 3, 1.8 and 0.6 in the presence of 15 $\mu$M, 70 $\mu$M, 360 $\mu$M and 2 mM bisG10 respectively. Concentration dependence of the inhibition of the maximal bound ryanodine is plotted in Fig. 3B. The curve indicates that the concentration of bisG10 causing half-maximum inhibition is 280 $\mu$M in the presence of 10 nM ryanodine. Fig. 3B also shows that the kinetic constants of the ryanodine binding were not significantly affected by the drug. Similar binding experiments were conducted in the presence of 100 nM [$^3$H]ryanodine. Under these conditions, SR vesicles

maximally bound 20 pmol ryanodine/mg protein with a rate constant of 0.046 $min^{-1}$. We found that the concentration of bisG10 inducing half-maximum inhibition was shifted from 280 $\mu$M in the presence of 10 nM ryanodine to 610 $\mu$M in the presence of 100 nM ryanodine.

## 4. DISCUSSION

BisG10 was primarly known as a muscle relaxant in whole muscle preparations [21]. Using vesicles inserted in planar bilayers, Garcia and Miller [15] reported that bisG10 was the most potent inhibitor of the SR $K^+$ channel. This was confirmed in stopped-flow fluorescence quenching experiments on SR vesicles where bisG10 was found to inhibit the rapid fluxes of monovalent cations which are thought to pass through the $K^+$ channel. In this study we found that bisG10 strongly inhibited the $Ca^{2+}$ release from SR vesicles induced by $Ca^{2+}$ and caffeine in the presence of extravesicular $K^+$ as counter-ions. This effect on the $Ca^{2+}$ release may however not be only interpreted in terms of a blockade of $K^+$ channels i.e. by stopping the $K^+$ counter-ion movement since (i) the $K^+$ ionophore valinomycin did not reverse the inhibitory effect of bisG10 and (ii) the $Ca^{2+}$ release elicited in the presence of $Cl^-$ as co-ions was still inhibited by bisG10.

A direct inhibitory action of the drug on the $Ca^{2+}$ release process was therefore tested by ryanodine binding experiments on SR vesicles. They gave evidence that bisG10 affects the SR $Ca^{2+}$ release channel. We found that bisG10 reduced the maximal amount of ryanodine bound to SR vesicles in a dose-dependent manner. Kinetic constants of binding were not modified by the drug and a 10-fold enhancement of ryanodine concentration only raised the half-maximum inhibiting concentration of bisG10 two-fold. Therefore, the ryanodine binding seems to be inhibited in a non-competitive way.

In the light of our findings, data obtained with bisG10 must be interpreted with caution. In their work on skinned fibers, Abramcheck and Best [11] postulated that the decreased $Ca^{2+}$ release rate observed in the presence of bisG10, in the same range of concentrations as we used, was due to a reduction of the $K^+$ counter-ion flow. They specified that to obtain a 50% decrease of release rate, about 270 $\mu$M bisG10 was needed, a value close to the concentration (280 $\mu$M) causing in our study half maximum inhibition of the ryanodine binding capacity of SR vesicles. We can thus postulate that, in the skinned fiber experiments of Abramcheck and Best [11], the decrease in $Ca^{2+}$ release rate observed arose, at least partially, from a direct inhibition of the SR $Ca^{2+}$ release channel.

Summing up, our results demonstrate that bisG10 exerts a blocking action on the SR $Ca^{2+}$ release channel with quite a good affinity. That bisG10 simultaneously blocks the SR $Ca^{2+}$ release channel and the SR $K^+$ channel is of particular interest. Channel blockers are often used as probes for the molecular structure of ion channels. The dual effect of bisG10 may suggest that both proteins display some structural homologies.

*Acknowledgements:* We are grateful to Dr. Guy Fournet from the Laboratoire de Chimie Organique I, Villeurbanne for synthesizing bisG10. This work was supported by the Centre National de la Recherche Scientifique. B.A. is a recipient of a fellowship from the Association Française contre les Myopathies.

## REFERENCES

[1] Lai, F.A. and Meissner, G. (1989) J. Bioenerg. Biomemb. 21, 227–246.
[2] Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Nature 339, 439–445.
[3] McKinley, D. and Meissner, G. (1978) J. Membr. Biol. 44, 159–186.
[4] Kometani, T. and Kasai, M. (1978) J. Membr. Biol. 41, 295–308.
[5] Miller, C. (1978) J. Membr. Biol. 40, 1–23.
[6] Coronado, R., Rosenberg, R.L. and Miller, C. (1980) J. Gen. Physiol. 76, 425–446.
[7] Labarca, P.R., Coronado, R. and Miller, C. (1980) J. Gen. Physiol. 76, 397–424.
[8] Labarca, P.R. and Miller, C. (1981) J. Membr. Biol. 61, 31–38.
[9] Somlyo, A.V., Gonzalez-Serratos, H., McClellan, S.G. and Somlyo, A.P. (1981) J. Cell. Biol. 90, 577–594.
[10] Moutin, M.-J. and Dupont, Y. (1988) J. Biol. Chem. 263, 4228–4235.
[11] Abramcheck, C.W. and Best, P.M. (1989) J. Gen. Physiol. 93, 1–21.
[12] Fink, R.H.A. and Stephenson, D.G. (1987) Pflügers Arch. 409, 374–380.
[13] Coronado, R. and Miller, C. (1980) Nature 288, 495–497.
[14] Coronado, R. and Miller, C. (1982) J. Gen. Physiol. 79, 529–547.
[15] Garcia, A.M. and Miller C. (1984) J. Gen. Physiol. 83, 819–839.
[16] Hasselbach, W. and Makinose, M. (1963) Biochem. J. 339, 94–111.
[17] Dupont, Y. (1977) Eur. J. Biochem. 72, 185–190.
[18] Vianna, A.L. (1975) Biochem. Biophys. Acta 410, 389–406.
[19] Pressman, B.C. (1976) Annu. Rev. Biochem. 45, 501–530.
[20] Meissner, G. and McKinley, D. (1976) J. Membr. Biol. 30, 79–98.
[21] Ozawa, H., Fukuda, H. and Goto, M. (1962) Yakugaku Zasshi 82, 1274–1277.