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Effect of the calcium-channel blockers on calcium accumulation in sarcoplasmic reticulum of skeletal muscle

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Vesicular fragments of sarcoplasmic reticulum isolated from rabbit skeletal muscle were actively loaded with Ca^{2+} in the presence of ATP and an ATP-regenerating system using Arsenazo III as metallochromic indicator to monitor Ca^{2+} movements across the membrane. Once the Ca^{2+} release is triggered by the presence of tetraphenylboron in the reaction medium, the addition of verapamil or diltiazem gives rise to a net Ca^{2+} entry inside the vesicles. Preincubation in the presence of verapamil does not abolish the tetraphenylboron-induced Ca^{2+} release, the verapamil-induced Ca^{2+} accumulation being still observed. There appears to be a high-affinity site for verapamil titrated in the micromolar concentration range, whereas diltiazem demonstrates more complex behavior when its concentration is raised. This study suggests the existence of a Ca^{2+} pathway (putative channels) which is blocked by the drugs tested allowing Ca^{2+} accumulation inside the vesicles owing to the Ca^{2+}-dependent ATPase activity.

Introduction

During skeletal muscle contraction, Ca^{2+} is released from the sarcoplasmic reticulum following an action potential. Few details are known about the molecular structures related with this event, although some of these studies have suggested that membrane proteins different from the Ca^{2+}-ATPase may be involved in gated Ca^{2+}-channels [1–3]. In fact, the existence of Ca^{2+}-channels in the plasma membrane of a number of excitable cells, including cardiac, smooth and skeletal muscle as well as neural and endocrine tissues, is well documented [4].

In this regard, extensive research has indicated the existence of a wide variety of compounds, the so called 'Ca^{2+}-channel blockers', which apparently exert their action through the voltage-dependent Ca^{2+}-channels [5,6]. These drugs are now used as major therapeutic agents because they relax vascular smooth muscle, providing vasodilation, and inhibit the slow inward Ca^{2+} current in cardiac muscle, producing a negative inotropic effect [7,8]. The structural diversity of the Ca^{2+}-channel blockers suggests that more than one site and mechanism of action exist. By using radioactive labelled Ca^{2+}-channel blockers in binding experiments, the presence of receptor-binding sites within the Ca^{2+}-channels has been demonstrated in different tissues corresponding to 1,4-dihydropyridine, diphenylalkylamine and benzothiazepine

Abbreviations: SR, sarcoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; TPB^-, tetraphenylboron.

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structures [4]. In skeletal muscle, the presence of Ca^{2+} channels has been inferred from binding experiments of tritiated Ca^{2+}-channel blockers [9,10]. Indeed, transverse tubule membranes are the most enriched source of Ca^{2+}-channels, providing excellent starting material for their solubilization and purification [11,12]. Nevertheless, since Ca^{2+} involved in skeletal muscle contraction is believed to come mainly from stores in sarcoplasmic reticulum, the existence of Ca^{2+}-channels in these intracellular membranes has been suggested from different experimental approaches [13–16], although at the moment no evidence is available concerning the influence of Ca^{2+}-channel blockers on those putative molecular structures. In the present study we show our results on isolated sarcoplasmic reticulum membranes obtained from rabbit hind leg white muscle which maintain a Ca^{2+}-pumping activity due to the presence of an ATP-regenerating system, studying the effect of the Ca^{2+}-channel blockers in relation to the process of Ca^{2+} release triggered by tetraphenylboron [17].

Materials and Methods

Chemicals. Arsenazo III sodium salt (approx. 98%), pyruvate kinase, creatine phosphokinase, (±)-verapamil and diltiazem were purchased from Sigma (U.K.).

Native SR vesicles. These were obtained from hind leg white muscle of female New Zealand rabbits as described by Eletr and Inesi [18].

Protein concentration. Protein was determined by the method of Lowry et al. [19] standardized with bovine serum albumin.

Estimation of Ca^{2+} concentration. Ca^{2+} in the reaction medium was estimated by the null-point titration method [20]. In our case, the absorbance was measured by dual-wavelength spectrophotometry (660–685 nm) using Arsenazo III as indicator.

ATPase activity. ATPase activity was assayed at 25 °C by measuring the amount of inorganic phosphate liberation using the molybdovanadate reaction [21]. The reaction medium contained: 50 mM Mops (pH 6.8), 80 mM NaCl, 10 mM $MgCl_2$, 50 μM total Ca^{2+}, 5 mM sodium phospho*enol*pyruvate, 10 IU/ml pyruvate kinase, 0.15 mg SR protein/ml and 100 μM Na_2-ATP. Once the hydrolytic reaction was initiated, 60 μM tetraphenyboron (at 8 min) and either 30 μM (±)-verapamil or 60 μM diltiazem (at 11.5 min) were added. All reagents were used as sodium salts because tetraphenylboron is not soluble in the presence of potassium.

Active Ca^{2+} loading. Ca^{2+} loading was performed in the presence of an ATP-regenerating system. Sarcoplasmic reticulum vesicles (0.15 mg/ml) were incubated at 25 °C in 50 mM Mops (pH 6.8), 80 mM NaCl, 10 mM $MgCl_2$, 25 μM Arsenazo III, 50 μM total Ca^{2+}, 5 mM sodium phosphocreatine and 20 μg/ml (approx. 3 IU/ml) creatine phosphokinase. The reactions were started by adding 100 μM Na_2-ATP.

Ca^{2+} release and reuptake. These were measured by differential absorbance changes of the metallochromic indicator Arsenazo III (660–685 nm) with the aid of an Aminco DW2a UV/Vis spectrophotometer, equipped with thermostatically controlled cell holder at 25 °C and with magnetic stirring. At the completion of loading, Ca^{2+} release was triggered by addition of 60 μM tetraphenylboron to the reaction medium and Ca^{2+} reuptake was elicited by the presence of (±)-verapamil or diltiazem. The timing of the additions was the same as for the ATPase activity experiments.

Results

Ca^{2+} release can be triggered from isolated sarcoplasmic reticulum or from sarcoplasmic reticulum of skinned muscle fibers by addition of the lipophilic anion tetraphenylboron, apparently by changing the membrane surface charge [17]. Thus, when 60 μM TPB^- was added at the completion of Ca^{2+} loading (about 200 nmol/mg protein) carried out in the presence of an ATP-regenerating system, Ca^{2+} release was immediately induced as measured by dual-wavelength spectrophotometry (Fig. 1A). This Ca^{2+}-release process (about 20 nmol/mg protein) corresponds to a rapid downward deflection (increase in absorbance) as a consequence of a net loss of Ca^{2+} by the sarcoplasmic reticulum vesicles until the system reaches again a new steady-state when the Ca^{2+} influx equals the Ca^{2+} efflux. In this new

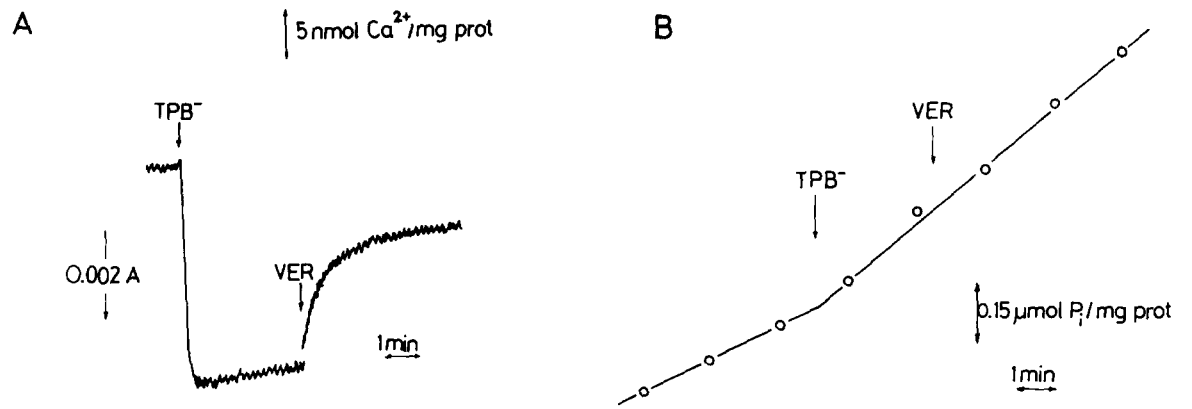

Fig. 1. (A). Ca^{2+} release and reuptake induced by TPB^- and verapamil, respectively. SR vesicles (0.15 mg/ml) were previously loaded in a medium containing 50 mM Mops (pH 6.8), 80 mM NaCl, 10 mM $MgCl_2$, 5 mM phosphocreatine, 20 μg/ml creatine phosphokinase, 25 μM Arsenazo III, 50 μM total Ca^{2+} and 100 μM ATP. At the completion of Ca^{2+} loading (8 min), 60 μM tetraphenylboron was added to evoked Ca^{2+} release. Ca^{2+} reuptake was elicited by addition of 15 μM (±)-verapamil (VER). Note that a downward deflection corresponds to an increase in absorbance and vice-versa. (B) Effect of TPB^- and (±)-verapamil (VER) on the ATPase activity coupled to the Ca^{2+} uptake mechanism. Ca^{2+}-ATPase was measured in the presence of an ATP-regenerating system as described in Materials and Methods. 60 μM TPB^- and 30 μM (±)-verapamil were added at 8 and 11.5 min, respectively, after the initiation of the hydrolytic reaction.

steady-state, the level of Ca^{2+} accumulated is lower and the subsequent addition of 15 μM (±)-verapamil, a Ca^{2+}-channel blocker of the diphenylalkylamines class, gives rise to a Ca^{2+} reuptake phase due to net Ca^{2+} entry inside the vesicles. No effect was observed in the absence of verapamil.

When the Ca^{2+}-ATPase activity was assayed under the same experimental conditions (Fig. 1B), the addition of 60 μM TPB^- to the reaction medium induced an activation of the hydrolytic activity rate in parallel with the Ca^{2+} release process evoked by this agent. Likewise, it is clearly shown that (±)-verapamil, in the concentration range used in this study, has no effect on the Ca^{2+} pumping mechanism carried out by the Ca^{2+}-ATPase.

Then, we investigated the effect of different verapamil concentrations upon the extent of Ca^{2+} reuptake (Fig. 2) keeping a constant ATP concentration in the reaction medium by using a phosphocreatine-creatine phosphokinase system. Under these conditions, the Ca^{2+} reuptake extent was dependent on the verapamil concentration, being approx. 17 nmol Ca^{2+}/mg protein the maximum reuptake response elicited by verapamil.

In order to prove whether verapamil can abolish the tetraphenylboron-induced Ca^{2+} release, experiments were performed in which racemic verapamil was added prior to the onset of Ca^{2+} release. The experimental trace illustrated in Fig. 3 reveals that tetraphenylboron in the presence of verapamil is still able to induce Ca^{2+} release. On the other hand, when SR vesicles were actively loaded in the presence of tetraphenylboron, the

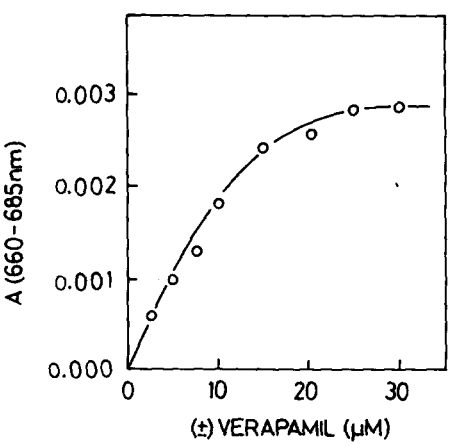

Fig. 2. Dependence of the extent of Ca^{2+} reuptake upon the verapamil concentration. Experiments were performed as described in Fig. 1A using the verapamil concentrations indicated on the abscissa.

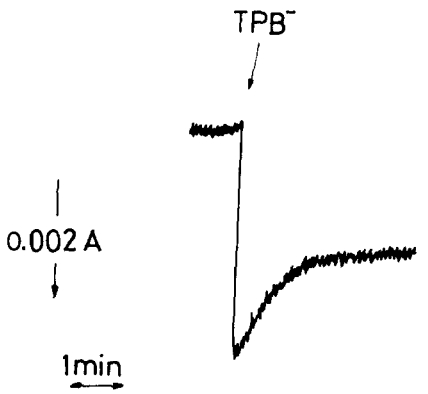

Fig. 3. Effect of TPB^- on sarcoplasmic reticulum vesicles loaded with Ca^{2+} in the presence of verapamil. SR vesicles (0.15 mg/ml) were previously loaded in the presence of: 50 mM Mops (pH 6.8), 80 mM NaCl, 10 mM $MgCl_2$, 5 mM phosphocreatine, 20 μg/ml creatine phosphokinase, 25 μM Arsenazo III, 50 μM total Ca^{2+}, 20 μM (±)-verapamil and 100 μM ATP. When the system reached steady state, 60 μM TPB^- was added to the reaction medium.

steady state was attained with a lower content of Ca^{2+} inside the vesicles when compared with the same experiment carried out in the absence of TPB^-; however, the addition of verapamil permits a net Ca^{2+} reuptake phase.

The time-course of Ca^{2+} uptake in the presence of verapamil is about the same as that in its absence. This suggests that verapamil blocks the rapid Ca^{2+}-release pathway, whereas the Ca^{2+}-uptake rate is dependent solely on the Ca^{2+}-pump activity in both cases.

We next studied the effect of diltiazem, a Ca^{2+}-channel blocker of the benzothiazepine class. As can be seen in Fig. 4A, an effect similar to that of verapamil upon the TPB^--induced Ca^{2+} release was obtained with diltiazem. Once the Ca^{2+}-release phase was completed and the system reached the steady state, the presence of diltiazem in micromolar concentration allows a transient Ca^{2+}-reuptake process to be monitored as the SR vesicles accumulate Ca^{2+}. In this regard, it should be noted that the verapamil concentration required to evoked Ca^{2+} reuptake was lower than that of diltiazem required to obtain the same response. The hydrolytic activity of the Ca^{2+}-ATPase during the Ca^{2+} release and reuptake induced by TPB^- and diltiazem, respectively, shows (Fig. 4B) an activation of the ATP hydrolysis induced by TPB^-, whereas diltiazem does not alter the enzymatic activity. The extent of the Ca^{2+}-reuptake phase as a function of the diltiazem concentration up to 70 μM is plotted in Fig. 5. The dependence observed for diltiazem is different to that presented by verapamil and hence it should be remarked that the use of diltiazem concentrations higher than 70 μM induces an apparent slow reuptake component, probably due to a more generalized effect of

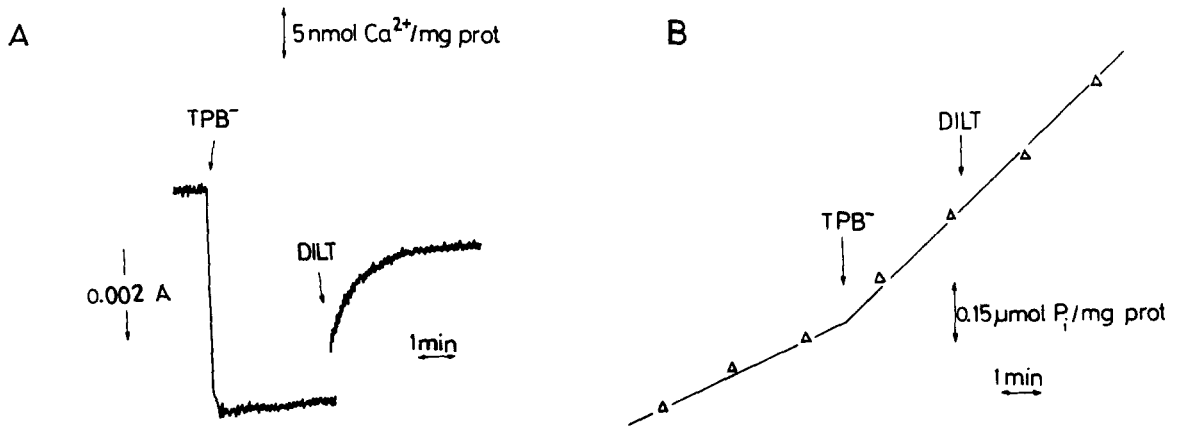

Fig. 4. (A) Ca^{2+} release and reuptake evoked by TPB^- and diltiazem (DILT), respectively. Ca^{2+} loading was previously carried out as described in Materials and Methods. Once the Ca^{2+} influx balanced the Ca^{2+} efflux, the response of the system to 60 μM TPB^- and 60 μM diltiazem was recorded. (B) Effect of TPB^- and diltiazem on the Ca^{2+}-dependent ATPase activity from sarcoplasmic reticulum. The hydrolytic activity of SR vesicles was measured as described in Materials and Methods. 60 μM TPB^- and 60 μM diltiazem were added at 8 and 11.5 min, respectively, after the initiation of the reaction.

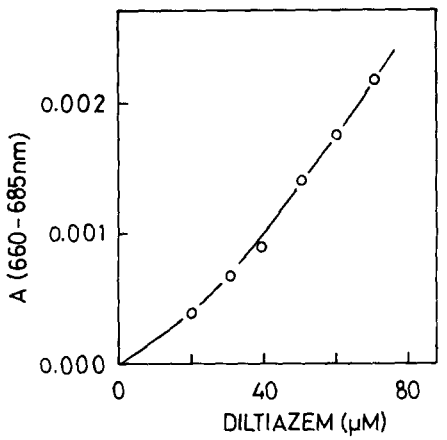

Fig. 5. Dependence of the extent of Ca^{2+} reuptake upon the diltiazem concentration. Experiments were performed as indicated in Fig. 4A but using diltiazem concentrations up to 70 μM.

diltiazem on the membrane. This behavior was not further investigated.

Fig. 6 illustrates the effect of two different diltiazem concentrations added after loading the sarcoplasmic reticulum vesicles in the presence of tetraphenylboron. In accordance with data reported above, 60 μM diltiazem gives the expected effect on Ca^{2+} reuptake, whereas 100 μM diltiazem induced a different response, showing a slow signal increase.

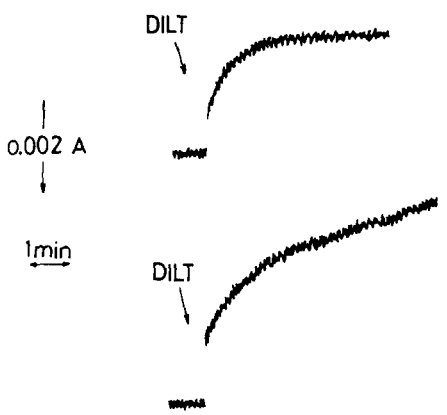

Fig. 6. Effect of diltiazem on SR vesicles loaded with Ca^{2+} in the presence of TPB^-. Active Ca^{2+} loading was previously performed in the presence of 60 μM TPB^-. Ca^{2+} uptake was induced by the addition of 60 μM (upper experiment) or 100 μM (lower experiment) diltiazem (DILT).

Discussion

One of the greatest difficulties associated with the characterization of macromolecules involved in electrical signaling of biological membranes is that they are usually present in a very low concentration, this being the case for the voltage-dependent Ca^{2+}-channels. Furthermore, the study of specific high-affinity binding sites is often hampered by nonspecific, low-affinity binding of ligands to membranes and even to the fact that the ratio of specific to nonspecific binding varies under certain experimental conditions. Therefore, an alternative approach to gain insight into these structures will be the study of their functional properties. Thus, we have conducted a number of experiments aimed at studying from a functional point of view the Ca^{2+} channels in sarcoplasmic reticulum by using two well-characterized Ca^{2+}-channel blockers, verapamil and diltiazem. For our purpose, SR vesicles were actively loaded with Ca^{2+} in the presence of ATP and an ATP-regenerating system, whereas changes in the extravesicular Ca^{2+} concentration were monitored by Arsenazo III, a Ca^{2+}-sensitive indicator dye. This procedure appears more gentle and less likely to lead to experimental artefacts than passive loading by extended preincubation of the vesicles in the presence of mM Ca^{2+} concentrations. In addition, the active loading method allows the participation of the Ca^{2+} channels, to be proved more clearly, since after blocking the channels, the presence of an active Ca^{2+}-pump will give rise to net Ca^{2+} reuptake.

It is known that Ca^{2+} release 'in vitro' can be evoked by a number of different methods [17,22–27], one of these being the presence of tetraphenylboron. Even though this method has no physiological relevance, it can be a useful tool along with the Ca^{2+}-channel blockers to characterize at the molecular level the mechanism involved in the process of Ca^{2+}-release from sarcoplasmic reticulum.

Titration of the verapamil effect on Ca^{2+} reuptake after TPB^--induced Ca^{2+} release reveals the existence of one high-affinity binding site for (±)-verapamil with an apparent K_d of approx. 8 μM under the experimental conditions used. On the other hand, when verapamil is added before

TPB^-, the release phase is still observed and only after Ca^{2+} is released a reuptake phase can be induced. This finding is consistent with the well-documented observation that verapamil and other diphenylalkylamines are specially effective in blocking Ca^{2+} channels that are open or depolarized, being therefore more effective where the frequency of channel opening and closing is greatest [28], as occurs in the sino-atrial and atrio-ventricular nodes of the heart. The simplest scheme to account for this experimental behavior where the channel blockers do not interact with closed or resting channels would be:

$$C_c \rightleftarrows C_o \overset{B}{\rightleftarrows} C_oB$$

being C_c the closed stated of the channel, C_o the opened state, B the blocking drug and C_oB the blocked state of the channel.

The data obtained for the reuptake phase in the presence of diltiazem are more complex to analyze. Initially, a proportional increase in the extent of the reuptake phase up to a certain diltiazem concentration can be observed; however, when the diltiazem concentration becomes higher, a slow reuptake component appears, making it difficult to assess this effect. Although these agents exert their actions primarily at the voltage-dependent Ca^{2+} channels, we can not rule out the possibility that they may have a significant effect on other ionic and receptor-mediated processes when relatively high concentrations are utilized. Finally, it should be noted that we were unable to elicit any blocking effect by using up to 100 μM nifedipine, a blocker of the dihydropyridine class. This might be a feature associated with the nature of this membrane, as we know that significant differences in affinity for the Ca^{2+}-channel blockers may exist in different tissues as a consequence of the heterogeneity of the Ca^{2+} channels. Thus, nimodipine, a blocker of the dihydroppyridine class has been found to select for vascular tissue in brain, whereas cinnarizine and other related compounds are more effective on smooth muscle than on cardiac muscle. In any case, the use of nifedipine must be always carefully controlled, since it needs to be dissolved in organic solvent, which may alter the membrane permeability. A further consideration is its yellow color, which may interfere with colorimetric measurements.

In conclusion, our studies provide the first evidence of the effect of Ca^{2+}-channel blockers on Ca^{2+} accumulation in a microsomal fraction enriched in longitudinal tubules of sarcoplasmic reticulum, showing that verapamil and diltiazem do not affect the enzymatic hydrolysis of ATP coupled to the Ca^{2+}-uptake mechanism; instead, they inhibit the TPB^--induced Ca^{2+}-release process, allowing accumulation of Ca^{2+} inside the vesicles due to the pumping activity of the Ca^{2+}-ATPase. It should be noted that microsomal fractions derived from terminal cisternae may show different functional properties as a consequence of their different origin in the intact muscle.

Acknowledgments

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