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Differential effects of organic calcium-channel blockers on diastolic SR calcium-handling in the frog heart

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1 Gradual loss of sarcoplasmic reticular (SR) calcium during a rest-period is responsible for the rest-induced decay (RID) of force in mammalian myocardium. Effect of verapamil and diltiazem on a similar RID in the frog myocardium suggests a new mechanism of action of these drugs.

2 Strips of frog-ventricle were paced at 0.2 Hz and the rhythm was interrupted by varying rest-periods ranging from 10 to 180 s. In control conditions, the amplitude of the post-rest beat was significantly lower than that of the pre-rest beat for rest-periods more than 40 s (RID).

3 Verapamil and diltiazem (which are organic calcium-channel blockers (OCCB)) changed the pattern of RID in the control solution to a 'rest-induced potentiation' (RIP) in the same preparation while another OCCB nifedipine and the inorganic calcium-channel blocker cadmium did not alter the post-rest phenomenon.

4 We propose that verapamil and diltiazem produce an RIP due to either blockade of SR calcium-leak during rest or enhancement of SR calcium-uptake during rest.
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Keywords: Diltiazem; frog-ventricle; nifedipine; rest-induced decay; rest-induced potentiation; sarcoplasmic reticular calcium; verapamil

Abbreviations: ECF, extracellular fluid; OCCB, organic calcium-channel blockers; RID, rest-induced decay; RIP, rest-induced potentiation; RyR, ryanodine receptors; SR, sarcoplasmic reticulum

Introduction

Organic calcium-channel blockers (OCCBs) like verapamil (a phenylalkylamine) and diltiazem, (a benzothiazepine) have long been used in the treatment of cardiac arrhythmias (Bigger, 1996) and another class of OCCBs, which are dihydropyridines (e.g. nifedipine, amlodipine) are used in the treatment of hypertension (Oates, 1996). The only known mechanism of action of OCCBs is blockade of sarcolemmal L-type calcium channels (Bean, 1985; Nilius *et al*., 1985). In the cardiac muscle, these drugs predictably produce a negative inotropism, but in the skeletal muscle, the OCCB diltiazem has been shown to have a paradoxical action – it increases twitch tension up to 80% over control (Gonzalez-Serratos *et al*., 1982). This action has not been well explained. The skeletal muscle does not depend on influx of calcium *via* sarcolemmal channels for contraction (Gonzalez-Serratos *et al*., 1982; Armstrong *et al*., 1972) and therefore it is understandable if diltiazem does not reduce force; but the enhancement of force is intriguing.

While working with strips of frog ventricle, we found that the preparation showed a rest-induced decay (RID) of force of contraction like the mammalian cardiac muscle. The RID was significant for rest-periods more than 40 s. Verapamil and diltiazem prevented such RID and in fact enhanced the force of contraction of the post-rest beat as compared to the pre-test beat (rest-induced potentiation, RIP). Based on our observations, we propose that, in addition to sarcolemmal calcium-channel blockade, verapamil and diltiazem either prevent a diastolic SR calcium-leak or augment a diastolic SR calcium-uptake.

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Methods

Frogs weighing between 70 and 110 g, belonging to the species *Rana hexadactyla* were used. Hearts isolated from pithed frogs were kept in ice-cold solution of the following composition (in mM per litre): NaCl 117, KCl 2, Na_2HPO_4 0.8, NaH_2PO_4 0.2, $CaCl_2$ 1 or 2, $MgCl_2$ 1, glucose 10, at a pH of 7.3–7.4. In some cases, a solution of otherwise similar composition, but containing 110 mM NaCl and 10 mM HEPES and devoid of the phosphates was used. There was no difference in the results when either buffer was used.

The atria of the isolated heart were removed and the apex of the ventricle was cut. The resulting circular ventricular strip was cut open. One end of the strip was anchored with silk to the base of a cylindrical bath, and the other end was connected to a force transducer. The force of contraction of the strip was recorded with a chart recorder. The bath was filled with a control solution, which was the same external solution as mentioned before, at a temperature of 25–28°C. The bath was continuously perfused and oxygenated.

HEPES was purchased from Loba Chemie and the other salts from MERCK. Diltiazem was a gift from Torrent Pharmaceuticals, verapamil was purchased from Torrent and nifedipine was a gift from CIPLA. Nifedipine stock solution was made by dissolving in ethanol.

Stimulus protocol

The ventricular strip described above was used to study the effect of rest on the post-rest contraction amplitude. The preparation was subjected to field-stimulation with metal electrodes (either stainless steel or chlorided silver) placed in the bath. It was continuously paced at a frequency of 0.2 Hz and allowed to stabilize for at least 45 min. After stabilization, the steady pacing was interrupted, and varying rest periods, ranging from 5 to 180 s, were imposed. The rest-periods were not given in a graded manner, but were randomized. Before a rest-interval was given, it was made sure that the force of contraction at 0.2 Hz stimulation had reached a steady state after recovery from the previous interval. The amplitude of the post-rest beat is expressed as a percentage of the pre-rest beat. If the post-rest beat was smaller than the beat just prior to the rest-interval, the phenomenon is referred to as RID. If the post-rest beat was larger than the pre-rest contraction, the phenomenon is referred to as RIP. The rest-protocol was employed and the relative amplitude of the post-rest beat studied before and after a drug of intervention. Only one drug or intervention was studied in one preparation. After the rest-protocol was done in control conditions, the calcium-channel blockers were allowed to act until the amplitude of contraction had steadied at the new lower level (a minimum of 15 min) before repeating the rest-protocol.

Expression and statistical analysis of data

Results are reported as mean ± standard deviation. Statistical significance of the negative inotropism of each calcium-channel blocker was assessed by two-tailed paired Student's *t*-tests done on steady-state amplitudes at 0.2 Hz before and after drug application.

For the experiments with the rest-protocol, the amplitude of the first post-rest beat is expressed as a percentage of the amplitude of the beat immediately preceding the rest-period. Statistical significance of the rest-induced decay with different rest-periods under control conditions was assessed by paired *t*-tests with a Bonferroni correction.

The influence of rest-periods on the relative amplitudes of the post-rest beats after a drug or intervention as compared to control conditions in the same preparation was assessed for significance by repeated measures ANOVA. Probability values of $P<0.05$ were considered significant.

Results

While beating steadily at 0.2 Hz in the control solution with 1 or 2 mM calcium, an intervening rest-period generally caused the post-rest contraction to have a smaller amplitude as compared to the pre-rest contraction in 31 out of 33 preparations tested. Such rest-induced decay was statistically significant for rest-periods more than 40 s ($P<0.05$, with paired *t*-tests with Bonferroni correction) and increased with increasing rest periods (Figure 1).

Negative inotropism of calcium-channel blockers

The inorganic calcium-channel blocker cadmium (10 μM), and the OCCBs verapamil (10 μM), diltiazem (10 μM), and nifedipine (1 and 10 μM) showed significant negative inotropism (Figure 2).

Cadmium chloride

The inorganic calcium-channel blocker cadmium, (Lee & Tsien, 1982) (as cadmium chloride, 10 μM) was negatively inotropic as expected for calcium-channel blockers (Figure 2). It did not alter the post-rest phenomenon significantly (Figure 3). The RID, which was observed in the control solution, was present even after perfusion of a solution containing 10 μM cadmium chloride. If anything, the magnitude of decay had only worsened after cadmium.

High ECF calcium

When calcium in the ECF was increased from 1 to 5 mM, positive inotropism was seen as expected. In addition, the RID was reversed to RIP. The rest-induced potentiation with 5 mM calcium persisted after cadmium blockade of the sarcolemmal calcium channels (Figure 4), though cadmium reduced the force of contraction.

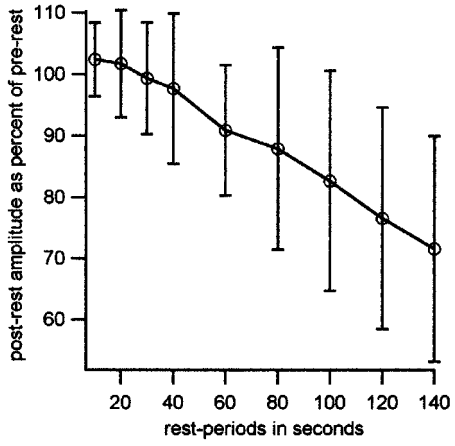

Figure 1 Rest-induced decay of force in strips of frog ventricle. Force of post-rest contraction is expressed as a percentage of pre-rest contraction. Each data point is mean ± s.d., $n=33$.

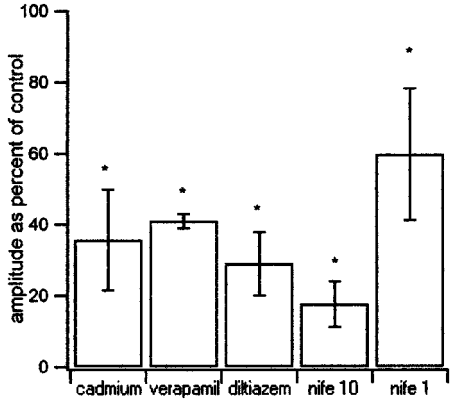

Figure 2 Effect of calcium-channel blockers on the steady-state force of contraction in isolated strips of frog ventricle. Values are mean ± s.d., $n=4-6$. $P<0.02$ in all cases with two-tailed paired *t*-tests.

Verapamil

Verapamil, an OCCB belonging to the group of phenylalkylamines, converted the RID in the control solution to RIP ($P<0.01$ with repeated measures ANOVA, $n=4$) at 10 μM concentration (Figure 5). The drug was nevertheless negatively inotropic (Figure 2).

Diltiazem

Another OCCB diltiazem, (10 μM) belonging to the group of benzothiazepines, in addition to being negatively inotropic, (Figure 2) converted the RID in the control solution to RIP ($P<0.005$, $n=4$) (Figure 6).

Nifedipine

Nifedipine is an OCCB belonging to the class of dihydropyridines. At 10 μM concentration, nifedipine was more negatively inotropic than verapamil or diltiazem (Figure 2). In 11 out of 11 preparations, nifedipine did not change the pattern of RID at this concentration ($P=0.32$ with repeated measures ANOVA) (Figure 7). At a concentration of 1 μM, nifedipine was less negatively inotropic than verapamil (10 μM) or diltiazem (10 μM), but still did not change the RID seen in control conditions in seven out of nine preparations (Figure 8) ($P=0.93$ with repeated measures ANOVA, $n=9$). The two preparations where nifedipine 1 μM suggested RIP have also been included in the analysis.

Discussion

Rest-induced decay of force of contraction is a well-documented phenomenon in the mammalian heart (Reiter, 1988) and is attributed to a steady leak of calcium from the SR during a rest-period (Reiter, 1988; Kitazawa, 1984).

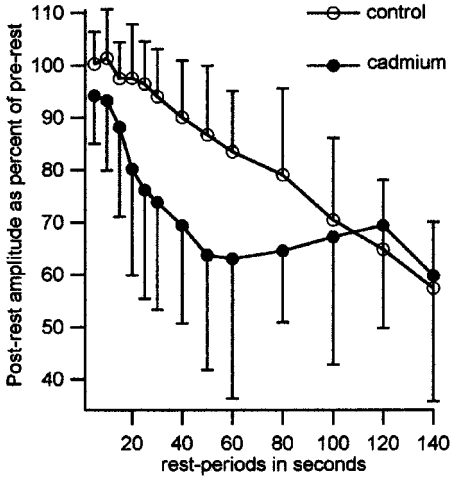

Figure 3 Rest-induced decay of force at 1 mM ECF calcium persists after cadmium blockade of sarcolemmal calcium-channels in strips of frog ventricle. Mean$\pm$s.d., $n=4$. $P>0.05$ with repeated measures ANOVA.

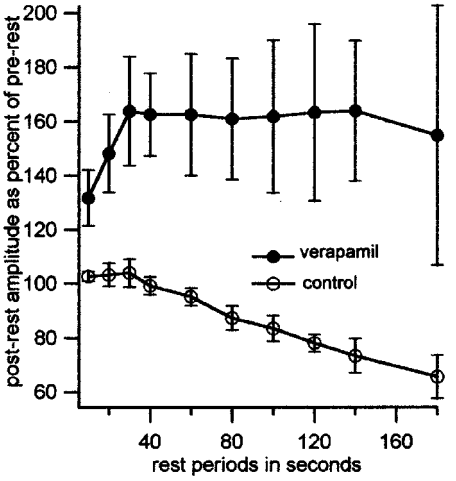

Figure 5 Verapamil 10 μM, converts RID to RIP in frog ventricular strips. Values are mean$\pm$s.d., $n=4$. $P<0.01$ with repeated measures ANOVA.

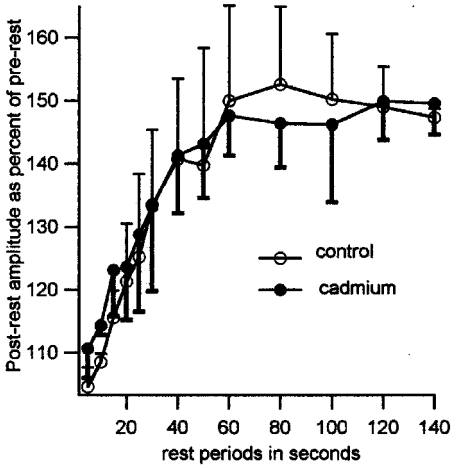

Figure 4 High ECF calcium (5 mM) produces rest-induced potentiation of force in frog ventricle, with or without cadmium 10 μM. Mean$\pm$s.d., $n=3$.

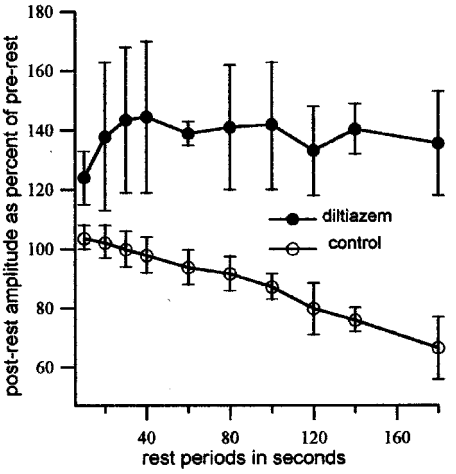

Figure 6 Diltiazem 10 μM, converts RID to RIP in strips of frog ventricle. (mean$\pm$s.d., $n=4$). $P<0.005$ with repeated measures ANOVA.

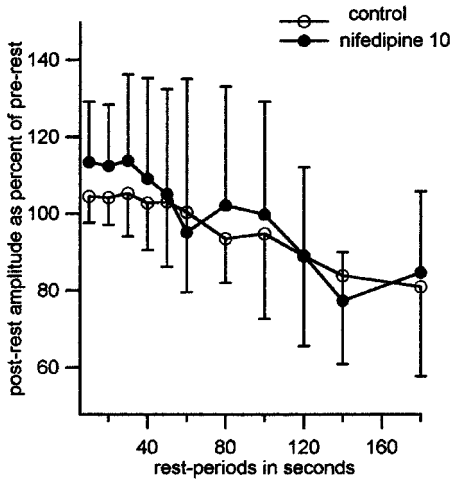

Figure 7 Nifedipine (10 μM) does not change the pattern of RID seen in control solution in frog ventricle. Values are mean$\pm$s.d. ($n=11$). $P=0.32$ with repeated measures ANOVA.

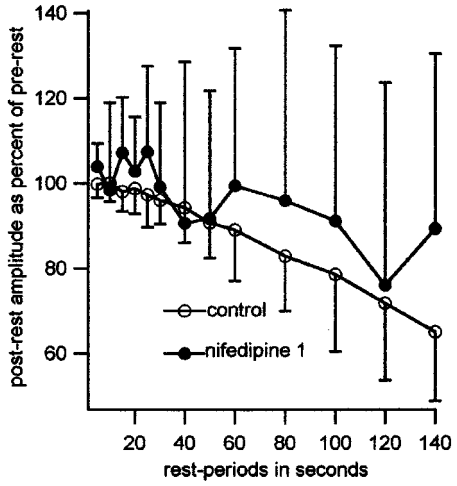

Figure 8 Nifedipine 1 μM does not change the pattern of RID in strips of frog ventricle. Values are mean$\pm$s.d., $n=9$. $P=0.93$ with repeated measures ANOVA.

We have shown that an RID occurs in the frog ventricle and that verapamil, diltiazem and high ECF calcium, can independently convert the RID to RIP. High calcium was positively inotropic and produced an RIP while the two OCCBs were negatively inotropic and still produced an RIP. The inorganic calcium-channel blocker cadmium, however, did not alter the RID with 1 mM ECF calcium or the RIP with 5 mM ECF calcium, though it was negatively inotropic in both cases. Nifedipine, an OCCB belonging to the class of dihydropyridines also did not alter the RID that was seen in the control solution at concentrations of 1 and 10 μM which produced less and more negative inotropism respectively, as compared to the other two OCCBs.

To explain these observations, we propose the following: Any difference in amplitude of the post-rest beat as compared to the pre-rest beat is solely due to a difference in the SR calcium content before and after rest. The difference in amplitudes of the beats before and after a rest-period is not attributable to a change in calcium influx *via* the L-type calcium-channels because, the observed difference in the amplitudes of pre and post-rest beats in the control solution exists even after cadmium blockade of the sarcolemmal calcium channels (Figures 3 and 4).

We also propose that the SR calcium content and therefore the contraction amplitude after a rest-period in the frog ventricle are dependent on a balance between two opposing events occurring during rest (analogous to the mammalian myocardium): a calcium leak from the SR (Kitazawa, 1984), and a calcium-uptake by the SR (Reiter, 1988). The leak pathway is not exactly clear. A ryanodine-insensitive calcium efflux pathway from SR has been reported. Such efflux could be *via* a specific calcium 'leak' pathway or due to a reversal of SR calcium pump (Duke & Steele, 2001).

The calcium-uptake during rest is *via* the sodium-calcium exchanger, which is sodium extrusive during diastole (Reiter, 1988; Lipp & Pott, 1988). This may seem controversial because one prevailing view is that the sodium-calcium exchanger is calcium extrusive. It is necessary to appreciate that the exchanger can take up calcium if the electrochemical gradient for sodium is reduced, especially if the calcium gradient is not reduced. The interval between contractions will therefore be most favourable for calcium uptake (Reiter, 1988). Lipp & Pott (1988) have observed that in isolated cardiac cells, the current due to the sodium-calcium exchanger is in the outward direction during rest at a holding potential of -50 mV, indicating a net sodium efflux, and therefore a calcium influx, given that the stoichiometry of the exchanger is 3 $Na^+ : Ca^{2+}$. The acquired calcium will enhance the loading of SR by the SR calcium-pump.

Our hypothesis is that in control conditions, the leak pathway is dominant and therefore the RID. But in the presence of high external calcium, verapamil or diltiazem, an RIP is seen because, either the leak is blocked or the uptake is augmented. It is easy to appreciate what might happen in the case of high ECF calcium. It may either block the leak by providing a steep gradient, which overwhelms the sarcolemmal calcium pump, or may enhance uptake *via* the sodium-calcium exchanger, or both.

In support of our hypothesis, we have observed that interventions, which are expected to augment sodium extrusion and therefore calcium acquisition by the sodium-calcium exchanger, have produced an RIP. 'Ouabain' (inhibits the sodium-pump and leaves the sodium-calcium exchanger as the main route of sodium extrusion), and 'low-sodium ECF solution' (containing 40 mM NaCl, the required osmolarity being maintained with sucrose), have produced RIP, in addition to different degrees of positive inotropism (unpublished observations).

The RIP produced by verapamil and diltiazem cannot be explained by their known mechanism of sarcolemmal calcium-channel blockade, because cadmium or nifedipine did not produce an RIP with 1 mM ECF calcium. Therefore verapamil and diltiazem must have another action, which could be any of the following: (1) Blockade of a putative leak channel on the SR membrane. If this is the mechanism by which verapamil and diltiazem act, the leak channel must be a different entity from the RyR (ryanodine receptor) channels, because the only source of calcium for contraction in the presence of OCCBs is the RyR channels (the L-type calcium channels having been blocked already). (2) Inhibition

of the sarcolemmal calcium pump, which may be the final mode of extrusion of the calcium that leaked from the SR. (3) Augmentation of calcium uptake by sodium-calcium exchanger during rest. (4) Augmentation of the SR calcium-pump.

It is highly probable that verapamil and diltiazem have an action on the SR membrane, because diltiazem has been shown to be positively inotropic in skinned skeletal muscle fibres (Gonzalez-Serratos *et al*., 1982). Positive inotropism in a skinned preparation is expected if diltiazem either blocks a leak channel on the SR membrane or enhances SR loading by the SR calcium-pump.

Differences in the actions of dihydropyridines and the other OCCBs are well known. Verapamil and diltiazem are known to produce bradycardia and atrioventricular conduction delay while nifedipine does not (Roden, 1996). This difference could be because verapamil and diltiazem block a diastolic SR calcium release, as we report here. Lipsius *et al*. (2001) have reported that a diastolic SR calcium release plays a role in atrial pacemaker function. This diastolic calcium-release stimulates inward sodium-calcium exchanger current to depolarize the pacemaker potential to threshold. On the basis of this mechanism, the authors suggest that interventions that alter SR calcium content and/or release are expected to regulate atrial pace-maker activity, in particular, that of latent atrial pace-makers. Inhibition of diastolic calcium release in the cells of the sinoatrial node, analogous to our observation on the frog-ventricle, may be the mechanism by which these two drugs cause bradycardia, and it is understandable that nifedipine does not cause bradycardia, because it does not prevent a diastolic SR calcium release. By the same action, verapamil and diltiazem may prevent ectopic atrial foci from firing and help to control supraventricular arrhythmias because the diastolic calcium-release from SR is particularly important for the late phase depolarization of latent atrial pacemakers (Lipsius *et al*., 2001).

'Over-riding an SR calcium leak' seems to be the most plausible hypothesis to explain the rest-potentiation seen with two diametrically opposite interventions, namely, verapamil and diltiazem, which are negatively inotropic, and high ECF calcium, which is positively inotropic. The opposite inotropic effects of these agents are due to their ability to block or enhance calcium influx through sarcolemmal calcium channels. In spite of differing actions on this channel, if both interventions can produce a rest-induced potentiation, such effect should be unrelated to sarcolemmal calcium channels.

In conclusion, the OCCBs verapamil and diltiazem, which are known to block L-type calcium channels on the sarcolemma may have an additional independent action of conserving SR calcium content during diastole either by blocking a diastolic SR calcium release or by enhancing calcium uptake.

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