Changes of Calcium Channel Inactivation during Run-down

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Abstract. Run down of calcium channel currents carried by Ba^{2+} was studied in enzymatically isolated ventricular cells from guinea-pig hearts using the suction pipette method. The decay of the Ba currents elicited by 100 ms voltage steps from −60 to 0 mV could be best described biexponentially. The peak current amplitude declined monotonously during dialysis of cell interior, whereas the inactivation showed a biphasic behaviour. After start of intracellular perfusion, the time constants of the fast and slow exponential component decreased up to the 6th and 10th minute, respectively. Thereafter the inactivation decelerated. These changes of the inactivation behaviour could be described by a model of current dependent inactivation. It was assumed, that in the first phase the decay is accelerated due to predominating of washout of phosphorylating agents and in the second phase the reduced conductance of the cell membrane for Ba^{2+} due to progressive irreversible closure of Ca channels causes slowing down of inactivation.

Key words: Ventricular myocytes — Voltage clamp — Ca channels — Inactivation — Run down

Introduction

It is well known that L-type Ca channel currents decline in amplitude if the solution at the inner side of the membrane is changed from the normal intracellular fluid to an artificial salt solution due to dialysis of the cell interior (Kostyuk et al. 1981; Fenwick et al. 1982) or to excision of cell patches out of the membrane (Nilius et al. 1985; Armstrong and Eckert 1987). This phenomenon has been called run-down or washout.

Several papers have been published showing that run-down can be slowed down, prevented or even reversed by Ca^{2+} chelating agents, calmodulin antagonists, ATP, cAMP, phosphodiesterase inhibitors, and catalytic subunit of protein kinase A (PKA) (Doroshenko et al 1984; Doroshenko and Martynyuk 1984; Byerly and Yazejian 1986; Chad and Eckert 1986; Belles et al. 1987; Belles

et al. 1988b). These results suggest that Ca^{2+}-dependent phosphorylation/dephosphorylation processes are involved in the washout of calcium channel currents.

Other experimental data indicate that dephosphorylation is also involved in the inactivation of Ca channels. Mg-ATP, 8-bromo-cAMP, ATP-τ-S or the catalytic subunit of PKA were found to slow down inactivation whereas the regulatory subunit of PKA, the kinase inhibitor protein or the Ca^{2+} dependent phosphatase calcineurin had the opposite effect (Byerly and Yazejian 1986; Eckert et al. 1986). These results support the model of current dependent inactivation of Eckert and Chad (1984); they proposed that the Ca^{2+} dependent dephosphorylation of the channel protein underlies the inactivation of Ca channels.

Only few data are available concerning the relation between run-down and inactivation of calcium channel currents. Here, we describe a relation between these two phenomena that are both supposed to be dependent on the phosphorylation of the calcium channel protein; the previously described model of current dependent inactivation (Standen and Stanfield 1982; Markwardt and Nilius 1988) will be used.

Materials and Methods

Voltage clamp experiments were carried out on enzymatically isolated ventricular cells from guinea pig hearts using the tight seal suction pipette method and intracellular perfusion. The dissociation procedure, the experimental setup, the fit of theoretical curves to the experimental data, as well as the calculations of the model of current dependent inactivation have been described elsewere (Benndorf et al. 1985; Markwardt and Nilius 1988). The cell interior was dialyzed with a solution containing (mmol l): 140 Tris-Cl, 2 EGTA, 2 $MgCl_2$, 5 Na-ATP; pH 7.1 titrated with H_3PO_4. The polished Pyrex glass pipettes had tip diameters between 2 and 6 µm. The extracellular bath solution consisted of (mmol l): 85 Cs-aspartate, 5 glucose, 5 Tris-Cl, 10 $BaCl_2$; pH 7.4 with aspartic acid and Tris. Ca channel currents with Ba^{2+} as the charge carrier were elicited every 5 s by 100 ms lasting voltage steps from a holding potential of -60 mV (to suppress the contribution of possible T-Ca-channel current) to a test potential of 0 mV.

Averaged statistical values are given as means $\pm$ standard error of the mean. Statistical significance of the correlation coefficient was estimated using Student's t-test (Weber 1980). P values less than 0.05 were considered significant.

Results

Figure 1*A* shows a typical example of calcium channel currents at different times after the start of the cell dialysis. Within 20 minutes the amplitude of the peak inward current declines monotonously. Also the mean peak values of the Ba

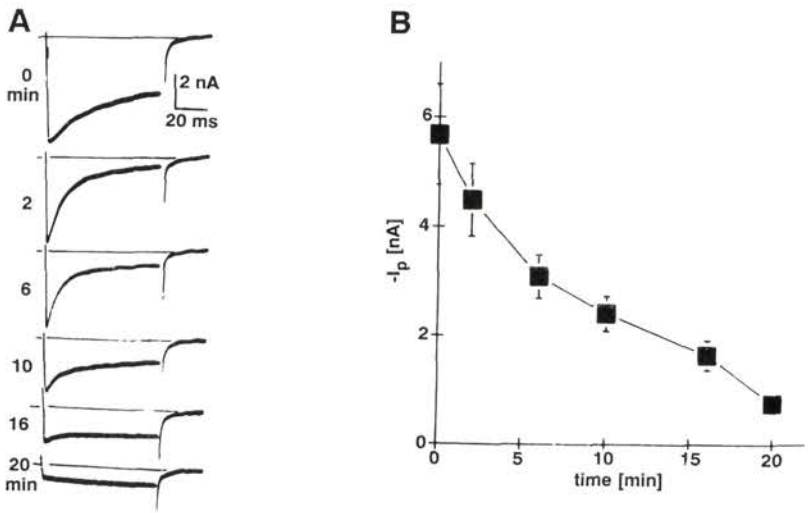

Fig. 1. *A*: Ba currents through L-type Ca channels in an enzymatically isolated ventricular cell from guinea-pig heart, evoked by 100 ms lasting voltage steps of 60 mV starting from a holding potential of −60 mV. The currents were measured at increasing intervals after the start of the cell dialysis (*left*). *B*: Mean values of peak inward Ba currents of 12 cells at different intervals after start of dialysis.

currents of 12 cells measured 0, 2, 6, 10, 16, and 20 minutes after starting intracellular perfusion of ventricular myocyte show a similar monotonous decrease (Fig. 1*B*).

Changes of inactivation during run down are more complex. Fig. 1*A* suggests that inactivation is speeded up during the first six minutes of dialysis, and slowed down thereafter. To provide support for this assumption and for a quantitative analysis the inactivation time course has been described by a mathematical model. The previously proposed test of Horn (1987) revealed two exponentially decreasing current components to be a significantly better fit of the inactivating part of our currents than is one exponential plus a pedestal, and insignificantly better than two exponentials plus pedestal. The decaying part of the currents could be described by:

$$I_{Ba} = I_1 . \exp(-a_1 . t) + I_2 . \exp(-a_2 . t), \tag{1}$$

where I_1 and I_2 are the amplitudes, and a_1 and a_2 are the reciprocal time constants of the fast and slow inactivating component, respectively.

Fig. 2*A* and *B* illustrate changes of a_1 and a_2 in time during washout. Up to the 6th (Fig. 2*A*) or 10th (Fig. 2*B*) minute after the start of the dialysis, respectively, a_1 and a_2 increase indicating a faster inactivation. Thereafter, the

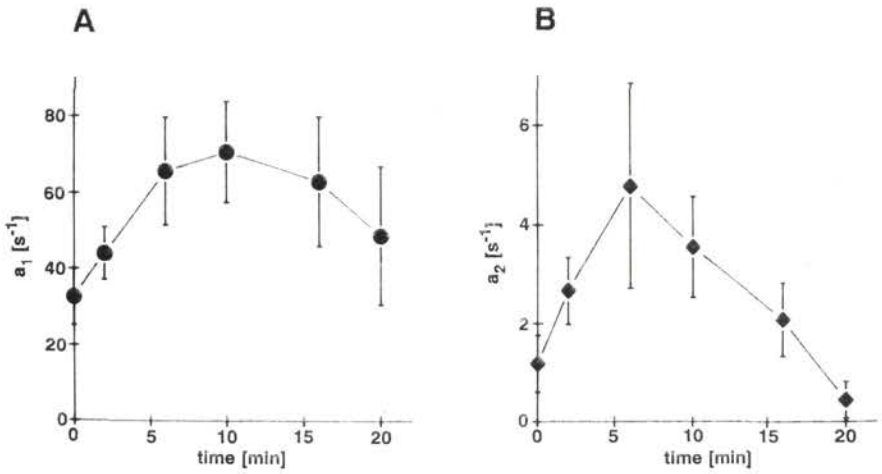

Fig. 2. The time dependence of the reciprocal time constants a_1 and a_2 of the fast (*A*) and slow (*B*) decaying exponential term, respectively (see Eq. 1). Mean values of 12 cells.

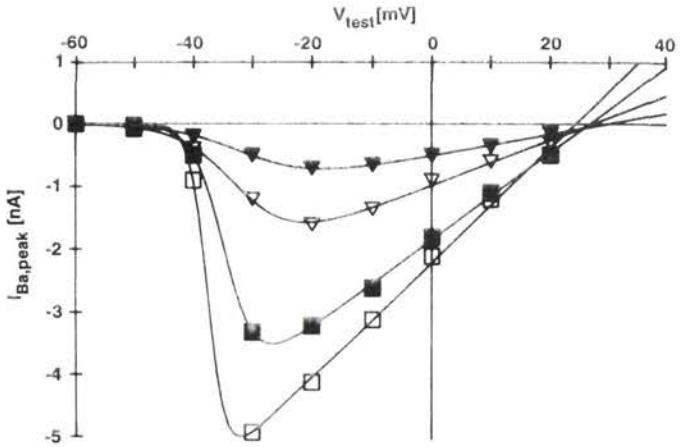

Fig. 3. The dependence of the voltage relationship of the peak Ba currents on duration of dialysis. The fits were done by

$$I_P = g_{max,\,Ba}(V_{rev} - V_t)/(1 + \exp((V_h - V_t)/s)), \tag{5}$$

where V_h is the potential of half maximal-activation, and s is the slope factor; for the meaning of $g_{max,\,Ba}$, V_t and V_{rev} see text. The values for the four intervals are shown in Table 2. The currents were elicited from a holding potential of -60 mV by 100 ms long test steps between -50 and $+20$ mV with 10 mV spacing. □ — start of dialysis; ■ — 8 min, ▽ — 22 min, ▼ — 34 min after start of dialysis.

inactivation decelerates in both cases. From 12 cells we found a significant positive time-correlation up to the 6th minute of dialysis. After this time the correlation was significantly negative.

The previously proposed model of current dependent inactivation (Standen and Stanfield 1982; Markwardt and Nilius 1988) has been expected to explain these results. The model assumes that Ba^{2+} ions, after passing the calcium channel, can be bound near the inner surface of the membrane thereby causing inactivation of the channel (for details see Markwardt and Nilius 1988):

$$Ba^{2+} + R^{2-} \underset{\alpha_f}{\overset{\beta_f}{\Leftrightarrow}} BaR. \tag{2}$$

A possible explanation for the inactivation speed-up could be a decrease in α_f due to an early reduction of the ability of the calcium channel protein phosphorylation. This mechanism would overlap with the slowing down of inactivation due to the decrease of the Ba currents during run-down. Therefore, we checked these assumptions by quantitatively describing of the measured currents by the Standen—Stanfield model.

The parameters of the Standen—Stanfield model were determined as follows:

For the sake of simplicity a d^2 activation scheme was assumed for the calcium channels, using the Hodgkin—Huxley formalism: the Ba currents were described by

$$I_{Ba} = g_{max,\,Ba} \cdot d^2(t) \cdot f(t) \cdot (V - V_R). \tag{3}$$

Neglecting the slow inactivation term, and setting zero the first derivation of Eq. 3 yields for the time to peak t_p

$$1/t_p = 1/(\tau_d \cdot \ln(2/(\tau_d \cdot a_1) + 1)). \tag{4}$$

A fit of the dependence of $1/t_p$ on a_1 for every measured current yielded an activation time constant of $\tau_d = 1.5$ ms which is in a reasonable agreement with the value obtained at the same test potential by Markwardt and Nilius (1988). The current-voltage-relationships of the Ba currents (Fig. 3) revealed $d_o = 0$ at the holding potential of -60 mV and $d_\infty = 1$ at the applied test potential of $V_t = 0$ mV. The extrapolated reversal potential V_{rev} was close to 30 mV. Values of the rate constant $\beta_f = 10\ mol^{-1} \cdot s^{-1}$, the cell volume of 40 pl, the volume fraction in which Ba^{2+} accumulates $\sigma = 0.2$, the reciprocal time constant for the first order removal of Ba^{2+} due to diffusion and dialysis $k_m = 0.005\ ms^{-1}$, and the fraction of Ba^{2+} unbound by EGTA $a = 0.3$ were adopted from Markwardt and Nilius (1988).

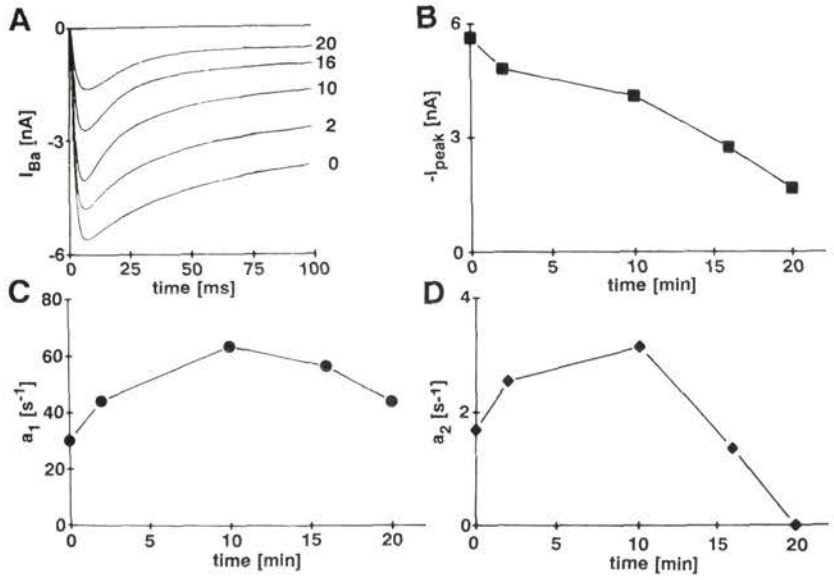

Fig. 4. The model of current dependent inactivation describes changes of peak current and inactivation during run-down. *A*: Simulation of Ba currents at different times of run-down, parameters see text. *B*: Peak values of the currents in *A*, compare with Fig. 1*B*. *C* and *D*: Reciprocal time constants a_1 and a_2, respectively, of the simulated currents shown in Fig. *A*, compare with Fig. 2.

All these parameters were kept constant. We tried to fit the measured dependences of the peak inward current I_P, a_1, and a_2 on the duration of the dialysis by changing only g_{max} and α_f, assuming a continuous decline of the cell Ba^{2+} conductance due to a progressive irreversible closure of Ca channels (number of functional channels decreases according to the terminology of Tsien et al. 1986) and to the reduced ability of the cell for phosphorylation because of washout of phosphorylating agents. Varying the parameters $g_{max,Ba}$ within 250 and 50 nS, and α_f within 1.5 and 0.02 ms^{-1} calculations of Ba currents were carried out using the Runge—Kutta-algorithm. These currents were fitted like the original traces using Eq. 1. The following set of parameters proved to best describe the averaged I_P, a_1, and a_2 of the measured currents at 0 (start), 2, 10, 16, and 20 minutes of dialysis:

Table 1. Parameters $g_{max,Ba}$ and α_f to fit the changes of the Ba currents observed during run-down

Time [min]	$g_{max,Ba}$ [nS]	α_f [ms^{-1}]
0	200	1.0
2	175	0.5
10	150	0.2
16	100	0.1
20	60	0.05

Table 2. Parameters of I_p—V-relation

Time [min]	$g_{max,Ba}$ [nS]	V_{rev} [mV]	V_h [mV]	s [mV]
0	91	24.2	−37.4	1.52
8	69	26.6	−34.4	2.66
22	36	27.1	−31.8	4.86
34	17	29.6	−30.0	5.98

Fig. 4 shows that with the above modifications the model is able to simulate the changes in current amplitude and inactivation observed during run-down. If Fig. 1*A*, 1*B*, 2*A*, and 2*B* are compared with Fig. 4*A*, 4*B*, 4*C*, and 4*D*, respectively, it becomes obvious that the model of current dependent inactivation can explain the principal changes of the time course of the measured Ba currents, the continuous decline of I_p, as well as the acceleration after the start and the deceleration of inactivation beyond the 10th minute of dialysis.

Discussion

Our study was designed to measure changes in inactivation due to run-down. The observed lifetime of Ba currents in our experiments (22 ± 3 min) is short in comparison to other reports (Eckert et al. 1986; Chad and Eckert 1986; Belles et al. 1988b). The likely reason for these quantitative differences might be the large tip diameter of our suction pipettes causing a stronger dialysis of the cell interior accompanied by a faster wash-out of phosphorylating agents than it is the case with whole-cell clamp pipettes. Calculating the diffusional exchange of substances through the pipette tip simply by

$$c(t) = c(0) \cdot \exp(-t/\tau) \tag{6}$$

we get a rough estimate of the time constant τ of diffusion of the regulatory subunit of PKA from the cell into the pipette (Pusch and Neher 1988). Using a molecular weight of 40,000 (Brum et al. 1983), the cell volume of 40 pl, and a mean access resistance of our pipettes $R_A = 0.4\ M\Omega$, a value of approximately 3 minutes is obtained for τ. The access resistances of approximately 4 $M\Omega$ as usual for whole cell recordings, yields a tenfold higher value. The introduction of the catalytic subunit of PKA in the dialysing fluid possibly would have slowed down the run-down.

Fig. 3 illustrates changes of the I—V-relationship during washout. It is obvious that the main changes concern the decrease of the slope conductance $g_{max,Ba}$. The rightward shift of the potential of half maximal activation by −7.4 mV, as well as the increase of the slope factor, may be caused by diminished voltage drop at the series resistance due to the decreased Ba current during run-down. This voltage error cannot be the reason for the measured changes of the inactivation behaviour, because within the range of test voltages close to 0 mV the changes of the inactivation time constants due to such voltage shifts are below than 10 % (data not shown, see Markwardt and Nilius 1988). Byerly and Yazejian (1986) also reported a positive shift of the potential of half maximal-activation comparable to our results, whereas Fenwick et al. (1982) found no major changes of the I—V-relationship during washout. Belles et al. (1988b) reported a negative shift of the steady-state activation curves by −9 mV during perfusion of the intracellular space. We were unable to exactly explain the reason for this contradiction; however, the shift in the extrapolated reversal potential points to the fact that the contribution of an outward current component to the leftward shift cannot be ruled out completely. Another explanation may be that the generation of a Donnan potential between the pipette solution and the cytoplasm, as discussed by Belles et al. (1988b), may be abolished with the suction pipette method because of the wider pipette tip diameters used in comparison to the whole-cell voltage clamp.

The main new experimental finding of our work is the biphasic change of inactivation during run down of calcium channel currents: inactivation is accelerated after the start of dialysis for several minutes, and is slowed down later. We consider two processes that may influence the speed of inactivation in opposite directions.

The first process, which, although continuously present, dominates at the beginning, is the progressive washout of phosphorylating agents, which reduces the ability of the myocyte to rephosphorylate the calcium channel. The idea of the involvement of rephosphorylation in the process of recovery from inactivation has been supported by the results of Eckert et al. (1986). These authors observed acceleration of the calcium channel recovery from inactivation induced by 8-bromo-cAMP an by the catalytic subunit of the protein kinase A, as well as an opposite effect of intracellular application of the regulatory subunit and inhibitors of PKA. Shimoni et al. (1987) observed an increase of the initial rate of reactivation by catecholamines and a slowing down of the recovery after acetylcholine administration. The exact reactions involved in the current dependent inactivation remain unknown. Thus, Eq. 2 may be only a simplificated scheme of the rate limiting step. Nevertheless, any decrease of the phosphorylating ability of the heart myocyte will shift the equilibrium of this simplified reaction scheme to the right, i.e. to the dephosphorylated and inactivated Ca

channel state. This causes a faster inactivation of all Ca channels which are phosphorylated previous to a depolarizing step.

The second process, which prevails during the later states, is irreversible closure of gradually more calcium channels wich are no longer available for opening by a depolarizing pulse. Such a behaviour of calcium channel currents was observed by Bechem and Pott (1985) who worked with dialysed guinea-pig atrial cardioballs (with EGTA, citrate and cAMP). Two effects may be responsible for this irreversible run-down. First, the interval of 4.9 s between two depolarizing pulses in our experiments may be too short for complete repriming, so that a cumulating number of Ca channels becomes not rephosphorylated. This effect may be amplified during run-down due to the continuous loss of phosphorylating agents. Second, Chad and Eckert (1986) reported about an irreversible component of run-down which depends on the activity of Ca activated proteases and which is independent of phosphorylation. These proteases might be calpain I and II, because their endogenous inhibitor calpastatin is able to delay run-down (Belles et al 1988a). Ba^{2+}, though in a much higher concentration, may also be able to activate these proteases. The irreversible washout results in a reduced number of Ca channels available for opening by depolarization of the cell membrane. This causes a reduced Ba^{2+} influx and a decreased Ba^{2+} concentration at the inner membrane surface which decelerates the current dependent inactivation.

Another possible explanation for the speeding up of inactivation during the first minutes of dialysis is that the buffering capacity of the cell for Ca^{2+} — and in parallel, although much lower, for Ba^{2+} — is reduced due to washout of buffering proteins. This hypothesis is supported by the findings of Byerly and Yazejian (1986). However, by increasing the fraction a of Ba^{2+} unbound by buffer, we were unable to simulate the strong biphasic changes of the decay of Ba currents observed in our experiments.

In conclusion, if the effects of certain experimental interventions (e.g., application of drugs) on the inactivation behaviour of whole-cell Ca channel currents is tested, the reversibility of the observed effects must be shown or, if impossible, the effect of the dialysis on the decay of Ca channel currents must be checked beforehand.

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