# EFFECTS ON L-TYPE CALCIUM CURRENT OF AGENTS INTERFERING WITH THE CYTOSKELETON OF ISOLATED GUINEA-PIG VENTRICULAR MYOCYTES

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## SUMMARY

We studied the effects of an external acute 10 min application of cytoskeletal interfering agents on cardiac L-type calcium current ($I_{Ca,L}$). We found that colchicine, taxol and cytochalasin D had no direct effect on the L-type calcium channel as indicated by the absence of effect on voltage-dependent parameters. Phalloidin induced a shift in the *I–V* curve which renders it difficult to use in excitation–contraction coupling studies. Microfilaments of actin did not seem to regulate cardiac $I_{Ca,L}$ as indicated by the lack of effect of cytochalasin D on $I_{Ca,L}$ amplitude and inactivation kinetics. On the contrary, microtubules seem to be involved in the calcium-dependent inactivation of $I_{Ca,L}$. This involvement might be direct, i.e. a physical link between the microtubules and some part of the channel protein, or it could be indirect, i.e. the calcium chelating properties and physical obstacle of microtubules in the space between the sarcolemma and the SR.

## INTRODUCTION

The cytoskeleton is known to play a role in organizing cell space (Lazarides, 1980) by restricting certain sarcolemmal proteins to specific domains within the cell (Li *et al.* 1993). The fact that the regulatory proteins of the cell are embedded within, and in many cases physically bound to, the cytoskeleton means that alterations in the cytoskeleton could affect the functioning of membrane proteins such as channels.

Some pharmacological tools exist which affect the different elements constituting the cytoskeleton. The effects of cytoskeletal stabilizers and disrupters on membrane channels have not been widely studied (for review see Cazorla *et al.* 1999). In different studies, these drugs have been applied using different methods: acutely using intracellular pipettes (Johnson & Byerly, 1993, 1994; Xu *et al.* 1997) or extracellular pipettes (Undrovinas *et al.* 1995; Xu *et al.* 1997), or over longer periods in the external solution during incubation either at room temperature (Maltsev & Undrovinas, 1997) or at 37 °C (Zhang *et al.* 1997). Acute application is the more direct method with which it has beeen shown that cytochalasin D, colchicine and taxol pass through the membrane and act on their respective target in less than 10 min (Galli & DeFelice, 1994; Undrovinas *et al.* 1995; Xu *et al.* 1997). Phalloidin is generally applied via intracellular pipette (Johnson & Byerly, 1993, 1994; Xu *et al.* 1997).

The effects of cytoskeletal stabilizers and disrupters on the L-type calcium current ($I_{Ca,L}$) are also unclear. Johnson & Byerly (1993, 1994) found that, in neuronal cells, phalloidin (a microfilament stabilizer) and taxol (a microtubule stabilizer) reduced the calcium-dependent inactivation and the run-down, taxol having the more pronounced effect; colchicine (a

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microtubule disrupter) and cytochalasin D (a microfilament disrupter) had no effect. Galli & DeFelice (1994) partly agreed finding that an acute external application of taxol also slowed the inactivation rate of $I_{Ca,L}$ but that colchicine increased it. They suggested that the cytoskeleton may regulate the concentration of $Ca^{2+}$ ions near the mouth of the channels.

Since the role of microtubules and microfilaments in the modification of $I_{Ca,L}$ has not been completely clarified, we studied the effects of phalloidin, taxol, cytochalasin D and colchicine on this current after 10 min acute external application at room temperature.

## METHODS

### *Single cell preparation*

Guinea-pigs (weight 250–400 g) were killed by cervical dislocation following stunning, according to national guidelines, and the hearts were removed. Single ventricular myocytes were isolated using collagenase and protease digestion as described elsewhere (Le Guennec *et al*. 1993). Isolated cells were placed in a 1·5 ml Perspex chamber on the stage of an inverted microscope (Diaphot, Nikon). The chamber was continuously perfused by gravity at a rate of 1–2 ml $min^{-1}$ with a 'standard' Tyrode solution containing (mM): 140 NaCl, 5·4 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 11 glucose, 0·33 $NaH_2PO_4$ and 10 Hepes (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid)), pH adjusted to 7·3 with NaOH.

### *Whole-cell current recording*

Single ventricular myocytes were voltage clamped using the whole-cell configuration of the patch-clamp technique (Hamill *et al*. 1981) with a RK-400 patch-clamp amplifier (Biologic, Grenoble, France). When the seal was reached and the whole-cell configuration established, the cell was submitted to the following external solution which contained (mM): 140 TEACl, 6 CsCl, 1·8 $CaCl_2$, 1 $MgCl_2$, 11 glucose, 10 Hepes, pH adjusted to 7·3 with TEAOH.

Borosilicate micropipettes had resistances between 2 and 5 MΩ and were filled with internal solution of the following composition (mM): 30 TEACl, 110 CsCl, 1 $MgCl_2$, 4 ATPMg, 11 Hepes and 10 ethyleneglycol bis-$\beta$-aminoethyl ether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH adjusted to 7·3 with CsOH.

Experiments were conducted and currents recorded using pCLAMP software (v.5.5.1) installed on a 386 SX 33 PC; currents were filtered at 3 or 10 kHz using a eight-pole Butterworth filter. The cell membrane capacitance was calculated by integrating currents elicited by 10 mV hyperpolarizing steps from a holding potential of −80 mV. In order to inactivate sodium current ($I_{Na}$) and transient T-type calcium current ($I_{Ca,T}$) a double-pulse protocol was used: a 500 ms depolarization from −80 mV to −40 mV was followed by a 500 ms depolarization from −40 mV to 0 mV. The double-pulse protocol was used every 8 s to allow a complete recovery of $I_{Ca,L}$ (Peineau *et al*. 1992). Long-lasting L-type calcium current ($I_{Ca,L}$) was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse. The protocol for constructing the *I–V* curve was as follows: from a holding potential of −80 mV, the membrane potential was stepped to potentials between −70 and +70 mV, in 10 mV increments, for 500 ms every 8 s. The lack of contamination of $I_{Ca,L}$ by $I_{Na}$ and $I_{Ca,T}$ is indicated by the lack of shoulder on the *I–V* curve at negative potentials (Lacampagne *et al*. 1994).

### *Drugs*

A cell attached to the pipette could be positioned at the mouth of any of four capillaries through which the different extracellular solutions were perfused by gravity.

Cytochalasin D, a microfilament disrupter, was dissolved in DMSO (dimethyl sulphoxide) as a stock solution (2 mM). Phalloidin, a microfilament stabilizer, was dissolved in distilled water as a stock solution (2·53 mM). Colchicine, a microtubule disrupter, was dissolved in ethanol as stock solution (50 mM). Taxol, a microtubule stabilizer, was dissolved in ethanol as stock solution (10 mM).

All substances and drugs were purchased from Sigma Chemicals (France).

### *Statistics*

To evaluate the shift induced by the chemical agents, the ascending part of each individual *I–V* curve was fitted to a Boltzmann function. The voltage at half-maximal activation was then compared before and after drug application.

Data are expressed as means ± S.E.M. (*n*, number of cells studied). Significance was determined using Student's paired *t* test; $P < 0·05$ was considered statistically significant.

Table 1. *Effects of agents that interfere with the cytoskeleton on the time constants of inactivation of* $I_{Ca,L}$

| | Colchicine | | Taxol | | Cytochalasin D | | Phalloidin | |
|---|---|---|---|---|---|---|---|---|
| | Control | Exp | Control | Exp | Control | Exp | Control | Exp |
| $\tau_{fast}$ (ms) | $15 \pm 3$ | $27 \pm 8$* | $11 \pm 4$ | $23 \pm 14$ * | $19 \pm 7$ | $26 \pm 9$ n.s. | $14 \pm 8$ | $17 \pm 6$ n.s. |
| $\tau_{slow}$ (ms) | $120 \pm 17$ | $140 \pm 18$ n.s. | $125 \pm 20$ | $118 \pm 17$ n.s. | $128 \pm 8$ | $140 \pm 28$ n.s. | $135 \pm 28$ | $108 \pm 31$ n.s. |

For each substance seven cells were studied. Significant differences ($P < 0{\cdot}05$) between time constants after 10 min of application (Exp) and just before starting the application (Control) are indicated by asterisks. n.s., non-significant. Each value is expressed in milliseconds and the means ± S.E.M. are given. The effects of all four substances on the time constants after 1 min of application were not signifcantly different from control and are not given.

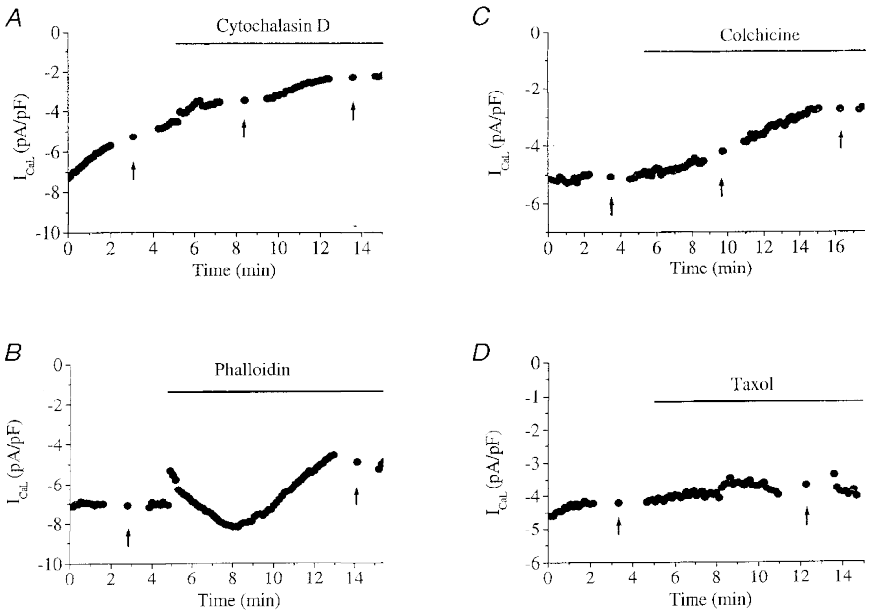


Fig. 1. Representative effects of substances acting on the cytoskeleton on the amplitude of $I_{Ca,L}$. Cytochalasin D (20 $\mu$M, *A*), phalloidin (50$\mu$M, *B*), colchicine (100 $\mu$M, *C*) and taxol (25 $\mu$M, *D*) were applied for 10 min as indicated by the bars. *I–V* curve protocols were performed at the points indicated by the arrows and the amplitude of $I_{Ca,L}$ when the cell is depolarized to 0 mV is given. Six other cells gave similar results for each substance.

## RESULTS

Acute application of cytochalasin D (20 $\mu$M), phalloidin (50 $\mu$M), taxol (25 $\mu$M) and colchicine (100 $\mu$M) was performed in groups of seven cells. As shown in Fig. 1, cytochalasin D, colchicine and taxol had no obvious effect on the amplitude of $I_{Ca,L}$ elicited by a voltage step to 0 mV during the 10 min of application. Phalloidin, however, first induced an immediate decrease in the current (within the 8 s separating the two voltage pulses) followed by an increase over 3 min and a decrease over the next 7 min. This behaviour was produced in all seven cells.

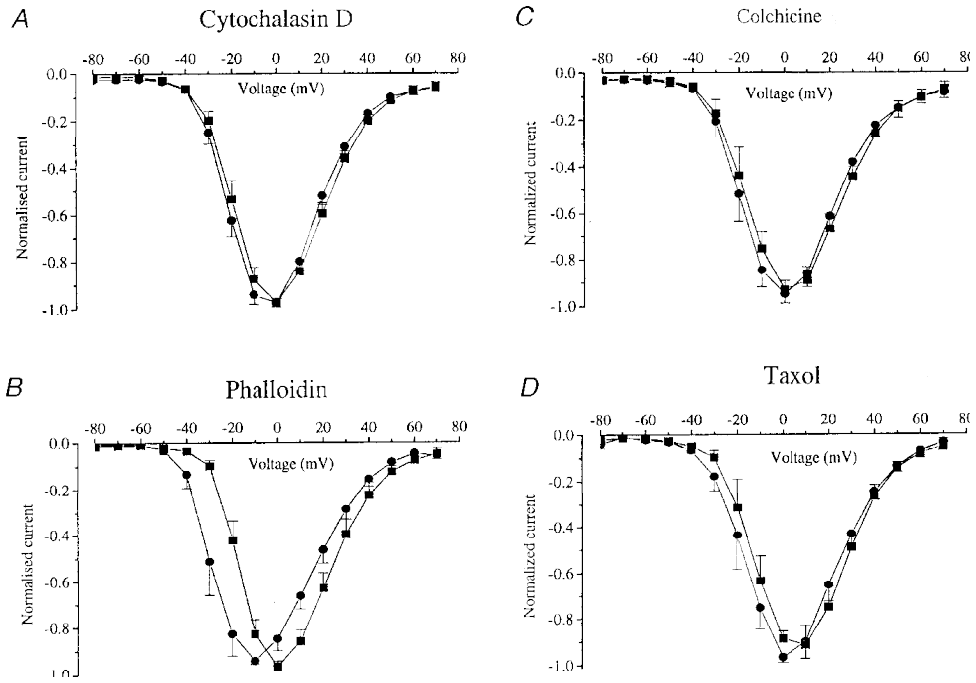


Fig. 2. Effects of substances interfering with the cytoskeleton on the *I–V* properties of $I_{Ca,L}$. For each cell, the current was normalized to the maximum current of each *I–V* curve (before (■) and after (●) application of the agent). The means ± S.E.M. are given from seven cells in cytochalasin D (*A*), six cells in phalloidin (*B*) and colchicine (*C*) and five cells in taxol (*D*). The only significant effect is the leftward shift obtained in presence of phalloidin (see text).

Examination of the normalized *I–V* curves (Fig. 2) shows that colchicine, cytochalasin D and taxol had no significant effect at all voltages. However, phalloidin induced a leftward shift of the *I–V* curve of 11 ± 5 mV ($P < 0{\cdot}01$).

Since some cytoskeletal interfering agents have been reported to affect the inactivation of $I_{Ca,L}$, we fitted the inactivation part of the current. The best fits were obtained using two exponentials. The two time constants ($\tau_{fast}$ and $\tau_{slow}$) in different experimental conditions are given in Table 1. The fast time constant was significantly affected by the agents acting on microtubules (colchicine and taxol), but not those on microfilaments (cytochalasin D and phalloidin). The slow time constant was not significantly affected by any of the drugs. Typical examples of the effects of these molecules on the inactivation phase of normalized $I_{Ca}$, are shown in Fig. 3.

## DISCUSSION

The aim of this work was to test the effects of acute application of some agents that interfere with the cytoskeleton on cardiac $I_{Ca,L}$ measured in the whole-cell configuration of the patch-clamp technique.

One of the problems with these molecules is their ability to cross the membrane and then act on the cytoskeleton. Galli & DeFelice (1994) reported that unitary $I_{Ca,L}$ recorded from chicken ventricular cells in the cell-attached configuration is regulated by microtubules as shown by its

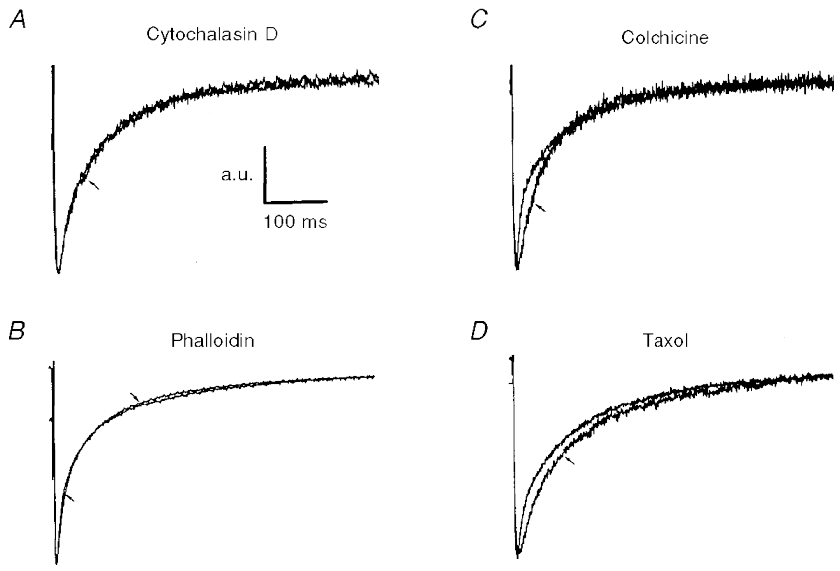


Fig. 3. Typical examples of the effects of substances interfering with the cytoskeleton on the inactivation phase of $I_{Ca,L}$. For clarity, currents have been normalized to the maximal inward current. Arrows indicate the trace in the presence of the molecule. a.u., arbitrary units.

sensitivity to 10 min external application of taxol or 15 min of colchicine. Undrovinas *et al.* (1995) reported that sodium current recorded from rat and rabbit heart cells was regulated by actin filaments as indicated by its sensitivity to acute application of cytochalasin D for 6 min. In the present study, the effects of cytochalasin D started to be visible at 2 min of application. Howarth *et al.* (1998) reported that cytochalasin D acted on contraction within 1 min following external application (probably by modifying the myofilament responsiveness to $Ca^{2+}$). These observations are evidence that cytochalasin D enters rapidly into the cell and that its effects probably last long enough (10 min) to modify actin microfilaments. Taxol and colchicine have also been shown to modify $I_{Ca}$ in opposite directions by Galli & DeFelice (1994). The effects were visible after 10 min external application and attributed to changes in microtubule architecture. Phalloidin, however, seems unable to pass through the membrane in such a short period of time. In general, people prefer to apply phalloidin intracellularly (Johnson & Byerly, 1993, 1994; Xu *et al.* 1997) or in incubation for several hours (Maltsev & Undrovinas, 1997).

Since agents that interfere with the cytoskeleton are very useful in cardiac excitation–contraction studies, it is important to know whether they act on $I_{Ca,L}$, which is the main means of entry for the calcium responsible for contraction (see Cleeman & Morad, 1991). To determine any possible direct effect on the channel, we first studied the voltage-dependent parameters.

The *I–V* curve and the slow time constant of inactivation, which is generally assumed to be solely modulated by the voltage (Carmeliet *et al.* 1986), are not affected by cytochalasin D, colchicine or taxol. The effects of phalloidin on $I_{Ca,L}$ amplitude are more complex. A leftward shift of the *I–V* curve was observed. In guinea-pig gastric myocytes, Xu *et al.* (1997) reported that 20 $\mu$M phalloidin applied via a patch pipette induced a rightward shift of the *I–V* curve of

a voltage-dependent calcium current. It seems that the direction of shift of the *I–V* curve depends on which side of the membrane the agent is applied. Phalloidin is a poorly charged cyclic oligopeptide. It is thus possible that it inserts into the membrane, displacing positive charges at the surface, which would be sufficient to shift the *I–V* curves. Whatever the origin of the shift of the *I–V* curve, it is clear that this effect renders the use of phalloidin difficult in excitation–contraction coupling studies.

It has been proposed that actin microfilaments and microtubules are involved in the calcium-dependent inactivation of a neuronal calcium current (Johnson & Byerly, 1993). To test this in cardiac cells, we fitted the current inactivation with two exponentials. At this point of the Discussion, it must be emphasized that addition of 10 mM EGTA to the patch pipette solution does not completely chelate intracellular calcium. As shown by Tseng (1988) and ourselves (Lacampagne *et al.* 1996; Brette *et al.* 1997), 10 mM EGTA is not enough to abolish calcium-induced calcium release just inside the membrane and that the cross-talk between the calcium channels of the SR and those of the sarcolemma still exists at this level. Thus, $Ca^{2+}$-dependent inactivation clearly occurs under our experimental conditions. Cytochalasin D and phalloidin have no effect on the time constants. Colchicine and taxol have a slowing effect on the rapid time constant. This time constant is assumed to be representative of the calcium-dependent inactivation (Carmeliet *et al.* 1986). The effects we found for taxol are thus in complete agreement with the effects found by Johnson & Byerly (1993) in neuronal cells and Galli & DeFelice (1994) in chicken cardiac cells. However, the effects of colchicine are in complete opposition since Galli & DeFelice (1994) found that colchicine speeds up the inactivation time course. They attributed the different effects of taxol and colchicine to local calcium buffering by microtubules: taxol would increase the density of microtubules and thus the calcium buffering power and colchicine would decrease the density of microtubules and thus the local $Ca^{2+}$ ion concentration would increase. We do not think, however, that a direct effect of colchicine on the L-type calcium channel of guinea-pig ventricular cells would explain this difference since the voltage dependence of the current is not changed by the microtubule disrupter. It is possible that a direct interaction between microtubules and the $\beta_2$ subunit of the L-type calcium channel is responsible for the inactivation of the channel, as already suggested (Chien *et al.* 1995). However, some fundamental structural differences between embryonic chick ventricular cells and guinea pig ventricular cells must be taken into account. Unlike guinea-pig ventricular cells embryonic chick ventricular cells do not have a sarcoplasmic reticulum (SR). We expect that the presence of microtubules could impede the ion diffusion in the space between the sarcolemma and the SR. If this is the case, a balance will exist between the chelating properties of microtubules, which would tend to decrease the local concentration of calcium, and the impedence of ion flow due to the physical presence of the microtubules, which would tend to increase the ionic concentration. With taxol, the increased density of microtubules would tend first to increase the concentration of calcium, then chelate it leading finally to a decreased local calcium concentration and thus a reduced inactivation of the current by calcium. With colchicine, the decreased density of microtubules would tend to increase the local calcium concentration but the reduction of the physical obstacle to ion diffusion could help calcium to decrease explaining the reduced inactivation of $I_{Ca,L}$ by calcium.

In conclusion, phalloidin should be used with care in excitation–contraction studies because it shifts the $I_{Ca,L}$–$V$ curve. The three other substances tested, colchicine, taxol and cytochalasin D, have no direct effect on the L-type calcium channel. Moreover, actin microfilaments do not appear to regulate $I_{Ca,L}$ (as shown here in guinea-pigs by the lack of effect of cytochalasin D

and in rat ventricular cardiac cells in Howarth *et al*. 1998) as they do for neuronal calcium current (Johnson & Byerly, 1993). Microtubules seem to be involved in the calcium-dependent inactivation of $I_{Ca,L}$. The involvement of microtubules is not clear. This complexity might be related to the presence of a SR when comparing these results with those obtained on cardiac cells lacking a SR (Galli & DeFelice, 1994).

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