Journal of Physiology (2002), **545.2**, *pp.* 375–388
DOI: 10.1113/jphysiol.2002.028605

www.jphysiol.org

Journal of Physiology

β-Adrenergic and muscarinic agonists modulate inactivation of L-type Ca^{2+} channel currents in guinea-pig ventricular myocytes

Ian Findlay

CNRS UMR 6542, Faculté des Sciences, Université de Tours, France

The objective of this study was to examine the effects of isoproterenol (isoprenaline) and carbachol upon voltage-dependent inactivation of L-type Ca^{2+} current ($I_{Ca,L}$). $I_{Ca,L}$ was recorded in guinea-pig isolated ventricular myocytes in the presence and absence of extracellular Ca^{2+} to separate total inactivation and voltage-dependent inactivation. In the presence of Ca^{2+}, isoproterenol and carbachol had 'competitive' effects upon the relationships between membrane voltage and $I_{Ca,L}$ amplitude and inactivation. Neither agonist had a marked effect upon the decay of inward $I_{Ca,L}$ carried by Ca^{2+}. In the absence of Ca^{2+}, isoproterenol severely reduced and slowed $I_{Ca,L}$ inactivation; this effect was reversed by carbachol. Under control conditions decay was dominated by fast inactivation. Isoproterenol reduced fast-inactivating and increased time-independent currents in a dose-dependent manner. These effects were counteracted by carbachol. There was a reciprocal relationship between the amplitude of fast-inactivating and time-independent currents with agonist stimulation. It is concluded that agonist modulation of rapid voltage-dependent inactivation of L-type Ca^{2+} channels involves an 'on–off' switch.

(Received 15 July 2002; accepted after revision 6 September 2002; first published online 18 October 2002)
Correspondence I. Findlay: CNRS UMR 6542, Faculté des Sciences, Parc de Grandmont, 37200 Tours, France.
Email: findlay@univ-tours.fr

The heart is subject to sympathetic and parasympathetic regulation, which is reflected in the responses of L-type Ca^{2+} channel currents ($I_{Ca,L}$) in isolated cardiac myocytes. $I_{Ca,L}$ is increased by β-adrenergic stimulation and subsequently reduced upon the application of muscarinic agonists (Trautwein & Hescheler, 1990). Inactivation is an important characteristic of $I_{Ca,L}$. It limits Ca^{2+} entry and contributes to the effective translation of electrical into mechanical activity. $I_{Ca,L}$ inactivates according to voltage- and intracellular Ca^{2+}-dependent mechanisms (Pelzer *et al.* 1990). The effect of β-adrenergic stimulation upon inactivation of $I_{Ca,L}$ in cardiac muscle varies between acceleration, no effect and slowing down, according to the report (Pelzer *et al.* 1990).

The mechanism of sympathetic and parasympathetic regulation of the cardiac muscle L-type Ca^{2+} channel involves the phosphorylation of one or more serine–threonine residues upon one or more of the channel subunits by protein kinase A (PKA) (Kamp & Hell, 2000). Interaction between the β-adrenergic and the muscarinic agonists occurs at different levels in the intracellular pathways leading to the activation of PKA, not at the level of the calcium channel (Kamp & Hell, 2000). Thus, if we are to understand the contribution of $I_{Ca,L}$ to the physiology of cardiac muscle it is necessary to understand the effects of phosphorylation by PKA upon the ion channel. Consideration that the L-type Ca^{2+} channel molecule or molecular complex might show distinct behaviour under basal conditions and following β-adrenergic stimulation is a surprisingly recent concept (Mitarai *et al.* 2000; Findlay, 2002*b*).

The consensus of opinion has been that Ca^{2+}-induced inactivation dominated the decay of cardiac muscle $I_{Ca,L}$ (Linz & Meyer, 1998), since voltage-dependent inactivation was exceedingly slow (Matsuda, 1986) and could not contribute significantly to the decay of $I_{Ca,L}$ under physiological conditions. In a pioneering study, Bean *et al.* (1984) showed that, whereas β-adrenergic stimulation accelerated the decay of inward currents carried by Ca^{2+}, in the same cells in the continued presence of Ca^{2+}, it slowed the decay of outward Ca^{2+} currents carried by Cs^{+}. Here, a clear dichotomy concerning the effects of β-adrenergic stimulation upon the inactivation of $I_{Ca,L}$ was revealed. In the first situation, the Ca^{2+} current would have been subject to total inactivation arising from Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum as well as membrane voltage. In the second situation, the Ca^{2+} current would have been subject to only voltage-dependent inactivation since, positive to the reversal potential, Ca^{2+} current would be carried by the efflux of intracellular monovalent cations (Tsien *et al.* 1987) and neither Ca^{2+} influx nor CICR would contribute to the decay. The importance of this observation was not appreciated, though several groups reported β-adrenergic

slowing of the decay of $I_{Ca,L}$ carried by Ba^{2+} (Tsien *et al.* 1986; Tiaho *et al.* 1991). Unfortunately, a large number of studies of either the influence of Ca^{2+} and CICR upon the inactivation of $I_{Ca,L}$ (Sham, 1997) or the voltage-dependent decay of $I_{Ca,L}$ (Matsuda, 1986) involved the use of particular experimental conditions to either impede 'run-down' or maximise currents, and/or to maximise interactions between the channel and sarcoplasmic reticulum, without taking into account that such treatments could alter the behaviour of the ion channel and thus bias the conclusions towards the importance of Ca^{2+}-induced inactivation.

In 1987, Hadley and Hume took advantage of the asymmetric permeation of the L-type Ca^{2+} channel to monitor the inactivation of $I_{Ca,L}$ in the absence of ion flux through the channels. When extracellular Ca^{2+} is removed, extracellular Mg^{2+} blocks inward but allows outward current through Ca^{2+} channels (Fukushima & Hagiwara, 1985; Hess *et al.* 1986). This outward current can be used to describe the inactivation of $I_{Ca,L}$ in the absence of Ca^{2+} and in the absence of inward ion flux where inactivation is a single and voltage-dependent process (Hadley & Hume, 1987; Hadley & Lederer, 1991; Findlay, 2002*b*). In this manner, Findlay (2002*b*) confirmed the observation of Bean *et al.* (1984) by showing that the decay of $I_{Ca,L}$ flowing outward through Ca^{2+} channels was slowed by β-adrenergic stimulation. However, in agreement with Mitarai *et al.* (2000), Findlay (2002*b*) showed that this was due to alteration of the kinetic make-up of the channel population rather than to a change in the process of inactivation. The principal aim of that previous study was to evaluate the relative contributions of voltage and Ca^{2+} to the decay of $I_{Ca,L}$ in isolated cardiac myocytes. It was shown that under basal conditions inactivation of $I_{Ca,L}$ depended largely upon membrane voltage while with β-adrenergic stimulation the reduction of the voltage-dependent kinetics of inactivation enabled the intervention of Ca^{2+}-induced inactivation. It was therefore clear that the relative contributions of these two processes to the overall decay of $I_{Ca,L}$ depended upon the kinetics of voltage-dependent decay and that this was the target of agonist stimulation of the channel. The objective of this present report was to further understanding of the effects of agonist stimulation upon voltage-dependent inactivation of the Ca^{2+} ion channel, by examining in some detail the influence of different doses of the β-adrenergic agonist isoproterenol (isoprenaline) and by testing, for the first time, the effects of the muscarinic agonist carbachol. The results revealed a surprising simplicity. Under basal conditions, the majority of Ca^{2+} channels show rapid voltage-dependent inactivation. In a dose-dependent manner, β-adrenergic agonists converted the channels to an inactivation-resistant form, whereas carbachol provoked a return to rapid voltage-dependent inactivation. It is therefore proposed that PKA phosphorylation of the L-type Ca^{2+} channel serves as an on–off switch for voltage-dependent inactivation.

METHODS

Cell preparation

All animal experiments were conducted according to the ethical standards of the Ministère Français de l'Agriculture (Licence number B37-261-4). Male guinea-pigs (250–400 g) were killed by cervical dislocation and the hearts were removed. Single ventricular myocytes were isolated using collagenase and protease digestion, as described elsewhere (Le Guennec *et al.* 1993). Myocytes isolated from the left ventricle were aliquoted into 35 mm diameter plastic Petri dishes that served as experimental chambers. The storage solution consisted of the standard extracellular solution described below. Dishes that contained myocytes were kept on the laboratory bench and used within 6–8 h after isolation.

Experimental procedures

Whole-cell current voltage-clamp experiments were conducted with an Axon Instruments 202A patch-clamp amplifier in resistive feedback mode (Axon Instruments, CA, USA). Pipettes were fabricated from thin-walled borosilicate glass capillary tubes (Clark Electromedical Instruments, Pangbourne, UK) with a Narishige PB7 double-stage puller (Narishige Instruments, Tokyo, Japan). Pipettes were coated with Sylgard (Dow Corning, MI, USA) and then heat polished. Finished pipettes had a resistance of < 2 MΩ when filled with standard intracellular solution. Plastic Petri dishes containing isolated myocytes were placed upon the stage of an Olympus CK2 inverted microscope. Isolated myocytes were superfused with experimental solutions via a parallel pipes system lowered into the vicinity of the cells. Experimental voltage-clamp protocols and data acquisition were controlled with Acquis1 software (Dipsi Industrie, Chatillon, France) installed upon a 386-20 PC computer. Data were filtered at either 1 or 2 kHz and acquired at 2 or 5 kHz, respectively. Cell capacitance and series resistance were compensated (~80 %) with the Axon Instruments amplifier. Cell currents are expressed as current density, pA pF^{-1}. Data analysis was performed with Acquis1 and Origin 4.1 (Microcal Software, MA, USA). Results are shown as mean ± S.E.M. values obtained from n different ventricular myocytes. Isolated myocytes were voltage clamped at –80 mV using the whole-cell configuration of the patch-clamp cell current recording technique (Hamill *et al.* 1981). Voltage-clamp protocols were delivered to the cells from this holding potential. Each voltage-clamp protocol was preceded by a voltage step to –50 mV for a period of 1000 ms to inactivate Na^+ current remaining after the application of 10 μM tetrodotoxin (TTX) and to inactivate any T-type Ca^{2+} current (Balke *et al.* 1992). The standard method of evoking cell currents consisted of a double-pulse voltage-clamp protocol with 1000 ms pre-pulse voltage steps to between −50 and +80 mV in 10 mV increments, and a 1000 ms duration test pulse voltage-clamp step to +80 mV. A 10 ms interval at −50 mV separated pre- and test pulse voltage steps. Current–voltage (I–V) relationships were established from the peak amplitude of currents evoked during pre-pulse voltage steps. Availability–voltage (A–V) relationships were established by normalising the amplitude of the current evoked by the test pulse to that evoked following a pre-pulse voltage step to −50 mV. The test pulse voltage step to +80 mV would evoke an outward $I_{Ca,L}$ carried by K^+ in experiments in which cells were bathed in normal extracellular solution containing 2 mM Ca^{2+} and also in

which cells were bathed in zero calcium extracellular solution (see Fig. 1). The amplitude of this outward $I_{Ca,L}$ served as a measure of the extent of inactivation occurring during the pre-pulse voltage steps. In this way, (1) the effects of evaluating inactivation of $I_{Ca,L}$ at different voltages would be avoided (Hadley & Hume, 1987; and see Gera & Byerly, 1999), (2) the test pulse current would not be subject to Ca^{2+}-induced inactivation whether recorded in the presence or absence of extracellular Ca^{2+}, and (3) direct comparisons could be made between curves recorded in the presence and absence of extracellular Ca^{2+} because extracellular Mg^{2+} in the zero calcium solution assured the maintenance of membrane surface charge (Findlay, 2002*b*). Voltage-gated currents through L-type Ca^{2+} channels were extracted from the ensemble whole-cell current with 200 μM $CdCl_2$. The limitations of this method are discussed by Linz & Meyer (1998). All experiments were conducted at room temperature (~23 °C).

Experimental solutions

The standard extracellular solution used to fill the Petri dishes and store myocytes prior to experiments contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4 with NaOH. TTX (citrate salt, 10 μM; from Alomone Labs, Jerusalem, Israel) and ryanodine (5 μM) were added to extracellular solutions used to superfuse cells during experiments. Calcium-free (zero calcium) extracellular solution contained 250 μM EGTA-NaOH, 3 mM $MgCl_2$ and no added Ca^{2+}. The standard intracellular solution used to fill the patch pipettes contained (mM): 140 KCl, 5 EGTA-KOH, 3.4 $MgCl_2$, 0.1 $CaCl_2$, 2 ATP-2Na^+, 0.1 GTP, 10 glucose, 10 Hepes, pH 7.3 with KOH. The estimated free concentrations of Mg^{2+} and Ca^{2+} in this solution were 1 mM and 1 nM, respectively. Isoproterenol and carbachol were prepared daily as 100 μM and 1 mM stock solutions, respectively, in distilled water. Ryanodine was dissolved as a 1 mM stock solution in distilled water.

RESULTS

Effects of agonists upon $I_{Ca,L}$

Figure 1 illustrates the effects of 100 nM isoproterenol and the subsequent addition of 10 μM carbachol upon $I_{Ca,L}$ recorded in the presence (Fig. 1*A–D*) and absence (Fig. 1*E–H*) of extracellular Ca^{2+}. Isoproterenol increased the amplitude of $I_{Ca,L}$ irrespective of whether this was an inward current carried by Ca^{2+} (Fig. 1*A–D*) or an outward current carried by K^+ in the presence (Fig. 1*A–D*) or absence (Fig. 1*E–H*) of extracellular Ca^{2+}. The addition of carbachol reduced the stimulation of $I_{Ca,L}$ induced by isoproterenol. Neither agonist altered the reversal potential of ~+55 mV between inwardly and outwardly directed currents through L-type Ca^{2+} channels (Fig. 1*D;* and see Bean *et al.* 1984).

The whole-cell current recordings shown in Fig. 1*A–C* and *E–G* illustrate the principal objectives of this study. The time-dependent decay of currents flowing thorough L-type Ca^{2+} channels was altered by agonist stimulation. In comparison with basal conditions (Fig. 1*A* and *E*), β-adrenergic stimulation provoked an apparent acceleration of the inactivation of $I_{Ca,L}$ carried by Ca^{2+} (Fig. 1*B,* inward current) while it apparently slowed down the inactivation of $I_{Ca,L}$ carried by K^+ (Fig. 1*B,* outward current, and *F*). These results are similar to previous observations (Bean *et al.* 1984; Tiaho *et al.* 1991; Mitarai *et al.* 2000; Findlay, 2002*b*). Here it is shown for the first time that the muscarinic agonist carbachol counteracted these effects of β-adrenergic stimulation. The inactivation of $I_{Ca,L}$ carried by Ca^{2+} (Fig. 1*C*, inward current) was apparently slowed by the application of carbachol in the continued presence of isoproterenol while the inactivation of $I_{Ca,L}$ carried by K^+ (Fig. 1*C*, outward current, and *G*) was accelerated.

The obvious difference between these effects of the agonists upon the time course of inactivation of $I_{Ca,L}$ was investigated in two stages. First, the effects of the agonists upon the total inactivation of $I_{Ca,L}$ were examined. These experiments were conducted upon ventricular myocytes which were bathed in extracellular solution containing 2 mM Ca^{2+}. Under these experimental conditions, inactivation of inwardly directed $I_{Ca,L}$ carried by Ca^{2+} would be due to both voltage- and Ca^{2+}-dependent processes (McDonald *et al.* 1994). Normally, Ca^{2+}-induced inactivation would result from both Ca^{2+} influx via cell surface channels and Ca^{2+} released from the sarcoplasmic reticulum. This last and variable component of the inactivation of $I_{Ca,L}$ (Adachi-Akahane *et al.* 1996; Hussain & Orchard, 1997; Sham, 1997; Findlay, 2002*b*) was excluded from this study by the inclusion of 5 μM ryanodine in all of the experimental solutions. Total inactivation of $I_{Ca,L}$ was therefore represented by voltage-dependent inactivation and Ca^{2+}-induced inactivation due to Ca^{2+} influx through L-type Ca^{2+} channels. Second, the effects of the agonists upon voltage-dependent inactivation was examined. These experiments were conducted upon ventricular myocytes bathed in extracellular solutions that did not contain Ca^{2+} but where Ca^{2+} had been replaced by Mg^{2+}. This method has been shown to adequately isolate the voltage-dependent process of inactivation of $I_{Ca,L}$ without disturbing the membrane voltage field (Findlay, 2002*b*; and see Hadley & Hume, 1987), which is a drawback of recording inward $I_{Ca,L}$ carried by Na^+ (Fukushima & Hagiwara, 1985; Mitarai *et al.* 2000; Findlay, 2002*a*).

The effects of agonists upon total inactivation of $I_{Ca,L}$

Figure 2 illustrates the effects of agonists upon the total inactivation of $I_{Ca,L}$, where this was represented by the quasi-steady-state availability curve recorded in normal extracellular solution containing 2 mM Ca^{2+}. Under basal conditions in the absence of agonists, the *A–V* relationship of $I_{Ca,L}$ was U-shaped with a half-inactivation voltage ($V_{0.5}$) of −20 mV (□, Fig. 2*A*). The application of isoproterenol enhanced inactivation of $I_{Ca,L}$ in a dose-dependent manner, such that $V_{0.5}$ was shifted to more negative values: −25 mV in 2 nM isoproterenol (■, Fig. 2*A*), −30 mV in 20 nM isoproterenol (■, Fig. 2*B*) and −29 mV in 100 nM isoproterenol (■, Fig. 2*C*). In each case recovery of $I_{Ca,L}$, which followed pre-pulse voltage steps to membrane potentials more positive than +20 mV, was enhanced compared with basal conditions. The addition of 10 μM

carbachol reversed the negative shift of the *A–V* relationship caused by 2 nM isoproterenol ($V_{0.5}$, −19 mV; ●, Fig. 2*A*), had a slight effect upon that recorded in the presence of 20 nM isoproterenol ($V_{0.5}$, −26 mV; ●, Fig. 2*B*), but had no effect upon that recorded in the presence of 100 nM isoproterenol ($V_{0.5}$, −30 mV; ●, Fig. 2*C*).

A–V relationships of $I_{Ca,L}$ evaluate inactivation at an approximation of steady-state conditions where it is assumed that the processes of voltage- and Ca^{2+}-dependent inactivation will have run their course. The influence of one or other of the processes may then be difficult to distinguish from the sum of their effects. In order to examine the relative influence of these processes, it is necessary to either examine the availability of the current at different and shorter intervals in time after activation of the current (Findlay, 2002*b*) or to follow the development of inactivation by recording the time course of decay of the current.

The effects of isoproterenol and carbachol upon the time course of decay of $I_{Ca,L}$ evoked by a voltage step to +10 mV were examined (Fig. 3). This voltage was chosen since it evoked nearly maximum inward Ca^{2+} current (Fig. 1*D*), was close to maximal to induce inactivation (Fig. 2), and was the highest voltage that in the absence of extracellular Ca^{2+} did not evoke current flow through L-type Ca^{2+} channels (Fig. 1*H*). This final point will be important for comparisons with subsequent experiments which were conducted in zero calcium extracellular solution (Fig. 5). Figure 3*A* illustrates $I_{Ca,L}$ recorded from four representative myocytes. Each recording consists of two cell current traces which were normalised to their respective peak amplitude and superimposed to allow a direct visual comparison of the time course of their inactivation. In the presence of extracellular Ca^{2+}, a voltage step to +10 mV evoked inward Ca^{2+} currents and carbachol was without discernable effect upon the time course of inactivation of $I_{Ca,L}$ recorded in the presence of three concentrations of isoproterenol. Carbachol induced a slight acceleration of the inactivation of $I_{Ca,L}$ recorded under basal conditions. The inactivation of $I_{Ca,L}$ evoked at +10 mV could be described by a double-exponential function (Origin 4.1), which under these circumstances declined to completion in a time-dependent manner. Isoproterenol increased the initial rate of inactivation of $I_{Ca,L}$ in a dose-dependent manner; τ_f = 25 ± 2, 17 ± 2, 17 ± 1 and 11 ± 1 ms under basal conditions and in the presence of 2, 20 and 100 nM isoproterenol, respectively. Neither isoproterenol nor

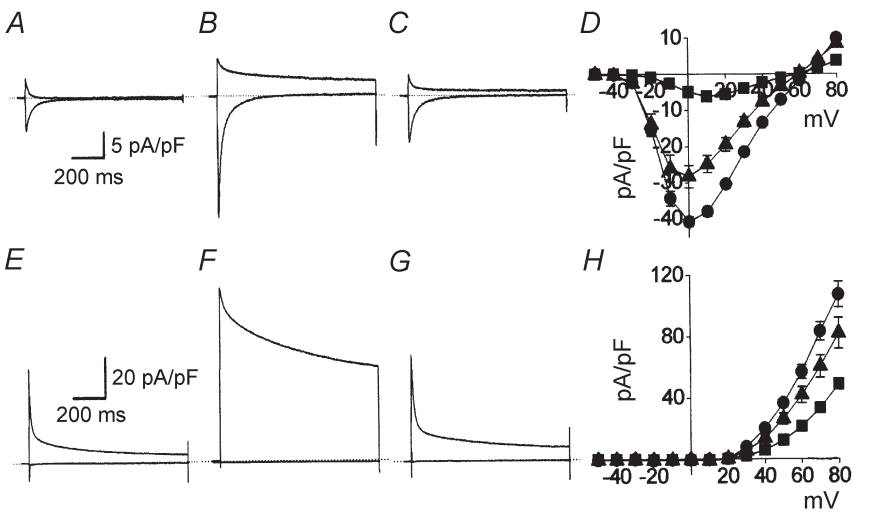

Figure 1. Effects of β-adrenergic and muscarinic stimulation upon $I_{Ca,L}$

Cell current records (*A–C* and *E–G*) represent superimposed cell current traces recorded during 1000 ms duration voltage steps to +10 mV (lower traces) and +80 mV (upper traces). The dotted lines indicate the 0 pA current level and the time and current scale bars in *A* and *E*, respectively, apply also to the records shown in *B* and *C*, and *F* and *G*. In one series of experiments (*A–D*), ventricular myocytes were bathed in extracellular solution containing 2 mM Ca^{2+}. *A*, cell currents recorded from a ventricular myocyte under basal conditions. *B*, cell currents recorded from a ventricular myocyte in the presence of 100 nM isoproterenol. *C*, cell currents recorded from the same myocyte following the addition of 10 μM carbachol. *D*, *I–V* relationships for $I_{Ca,L}$ recorded in the presence of 2 mM Ca^{2+} under basal conditions (■, n = 14), in the presence of 100 nM isoproterenol (●, n = 7) and following the addition of 10 μM carbachol (▲). In a separate series of experiments (*E–H*), ventricular myocytes were bathed in extracellular solution that did not contain Ca^{2+} (see Methods). *E*, cell currents recorded from a ventricular myocyte under basal conditions. *F*, cell currents recorded from a ventricular myocyte in the presence of 100 nM isoproterenol. *G*, cell currents recorded from the same myocyte following the addition of 10 μM carbachol. *H*, *I–V* relationships for $I_{Ca,L}$ recorded in the absence of extracellular Ca^{2+} under basal conditions (■, n = 8), in the presence of 100 nM isoproterenol (●, n = 8) and following the addition of 10 μM carbachol (▲).

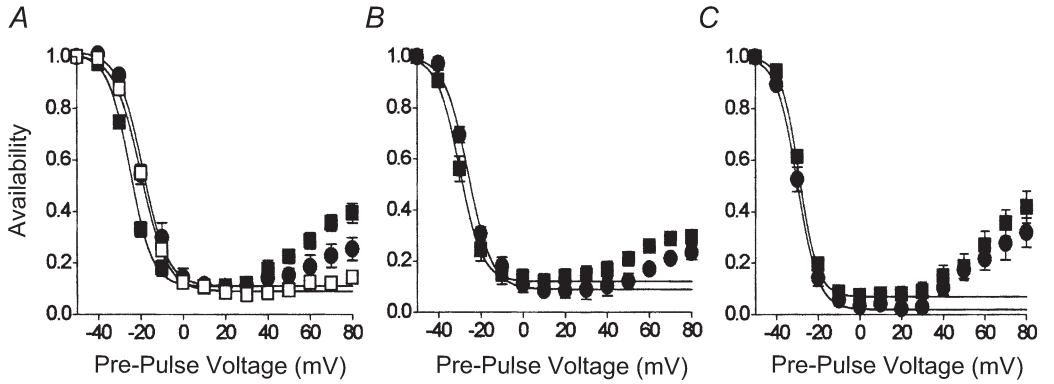

Figure 2. Effects of agonists upon the availability of $I_{Ca,L}$

These experiments were conducted in extracellular solution containing 2 mM Ca^{2+}. *A–V* relationships were recorded with the double-pulse voltage-clamp protocol described in Methods. *A*, effects of 2 nM isoproterenol (■, $n = 9$) and 10 μM carbachol (●). For comparison, data obtained from cells under basal conditions (□, $n = 14$) are also shown. *B*, effects of 20 nM isoproterenol (■, $n = 6$) and 10 μM carbachol (●). *C*, effects of 100 nM isoproterenol (■, $n = 7$) and 10 μM carbachol (●). The lines represent fits of the Boltzmann equation (Origin 4.1) between the maximum and minimum data points, and which were then extrapolated to +80 mV. See text for further details.

carbachol had any significant effect upon the relative proportions of the fast and slow components of biphasic decay of inward $I_{Ca,L}$ (Fig. 3*B*). It should be emphasised that these experiments were conducted in the presence of ryanodine in order to exclude any influence of Ca^{2+} release from the sarcoplasmic reticulum upon the inactivation of $I_{Ca,L}$. These results therefore show that neither agonist had a particularly marked effect upon the time course of inactivation of $I_{Ca,L}$ when carried by Ca^{2+} and when it was subject to both inactivation associated with membrane voltage and that caused by the influx of extracellular calcium. A different picture was to emerge from experiments conducted in the absence of extracellular Ca^{2+}.

The effects of agonists upon the voltage-dependent inactivation of $I_{Ca,L}$

The cell current records shown in Fig. 4 illustrate the effects of agonists upon the inactivation of $I_{Ca,L}$ occurring in the absence of current flow through the channels. Under basal conditions, the test pulse voltage step to +80 mV delivered from a pre-pulse voltage of –50 mV evoked a large outward and rapidly decaying current through L-type Ca^{2+} channels (Fig. 4*A*). When this voltage step was delivered after a 1000 ms pre-pulse voltage step to +10 mV, the outward current was severely reduced (arrow in Fig. 4*A*). No current had flowed through L-type Ca^{2+} channels during the pre-pulse voltage step to +10 mV. It is

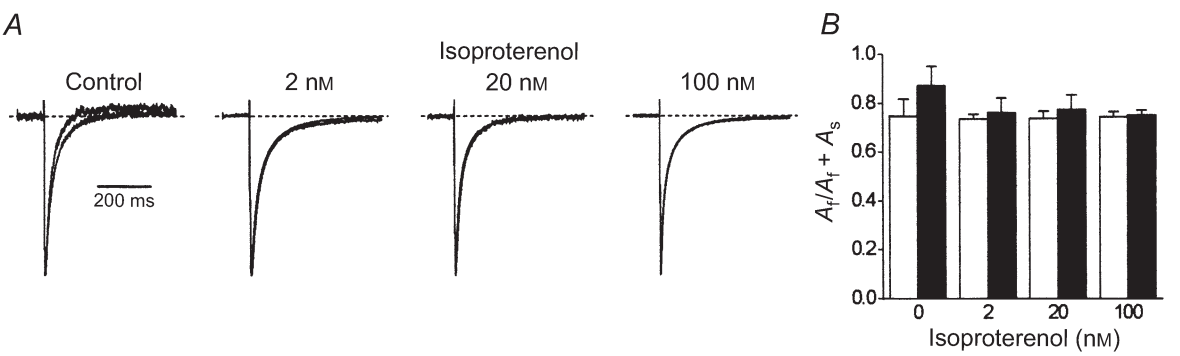

Figure 3. Effects of agonists upon the time-dependent decay of $I_{Ca,L}$

A, $I_{Ca,L}$ was evoked by voltage steps to +10 mV in cells bathed in extracellular solution containing 2 mM Ca^{2+}. These normalised records were obtained from four representative cells. Each record consists of two superimposed traces which were recorded first under the indicated experimental condition and then following the addition of 10 μM carbachol. The dotted lines indicate the zero level. The time scale to the left applies to all four sets of traces. *B*, the biphasic decay of $I_{Ca,L}$ evoked by a voltage step to +10 mV was analysed and the amplitudes of the fast and slow components of the double-exponential function (Origin 4.1) were extracted. Here, the amplitude of the fast component of decay is expressed as a proportion of the total current amplitude ($A_f/A_f + A_s$). Cell currents were recorded first either under basal conditions ($n = 8$) or in the presence of 2 ($n = 9$), 20 ($n = 6$) or 100 nM ($n = 5$) isoproterenol (□) and then following the addition of 10 μM carbachol (■).

concluded that $I_{Ca,L}$ had been activated and then inactivated during the voltage step to +10 mV in the absence of ion flux and that in consequence few L-type Ca^{2+} channels remained available for activation by the test pulse voltage step. The full range of the *A–V* relationship for $I_{Ca,L}$ recorded in the absence of extracellular Ca^{2+} under basal conditions is shown in Fig. 5*A* (□). This relationship was sigmoid and could be described by the Boltzmann equation over the entire voltage range between −50 and +80 mV tested here, and half inactivation ($V_{0.5}$) was assessed to have occurred at −20 mV. These results strongly suggest that, under these experimental conditions, with cells bathed in zero calcium extracellular solution, $I_{Ca,L}$ inactivated via a single and voltage-dependent process (Hadley & Hume, 1987; Findlay, 2002*b*).

The effect of different concentrations of isoproterenol upon the voltage-dependent inactivation of $I_{Ca,L}$ recorded in the absence of extracellular Ca^{2+} is shown in Fig. 4*B* and *C*. These recordings differed from the results obtained under basal conditions (Fig. 4*A*) in two ways. First, $I_{Ca,L}$ evoked by the test pulse voltage step showed less inactivation in isoproterenol in a dose-dependent manner (Fig. 4*B* and *C*). Second, the amount of $I_{Ca,L}$ remaining available for activation following a pre-pulse voltage step to +10 mV was increased by isoproterenol in a dose-dependent manner (arrows in Fig. 4*B* and *C*). These two observations strongly suggest a slowing and/or an inhibition of the process of voltage-dependent inactivation of $I_{Ca,L}$ by β-adrenergic stimulation. Figure 5 illustrates the *A–V* relationships for $I_{Ca,L}$ recorded in the absence of extracellular Ca^{2+} following stimulation by 2 nM (■, Fig. 5*A*), 20 nM (■, Fig. 5*B*) and 100 nM (■, Fig. 5*C*) isoproterenol. Isoproterenol had little effect upon the voltage dependence of the *A–V* relationships recorded in the absence of Ca^{2+}, with $V_{0.5}$ values of −22 mV in 2 nM isoproterenol, −20 mV in 20 nM isoproterenol and −21 mV in 100 nM isoproterenol, compared with –20 mV for $V_{0.5}$ recorded under basal conditions. However, isoproterenol clearly reduced the amount of inactivation recorded over 1000 ms in a dose-dependent manner. Thus ~40 % of the total cell current in 2 nM isoproterenol and ~70 % of the total cell current in 20 and 100 nM isoproterenol remained available for activation at the minima of the *A–V* relationships compared with only ~6 % of the total cell current under basal conditions.

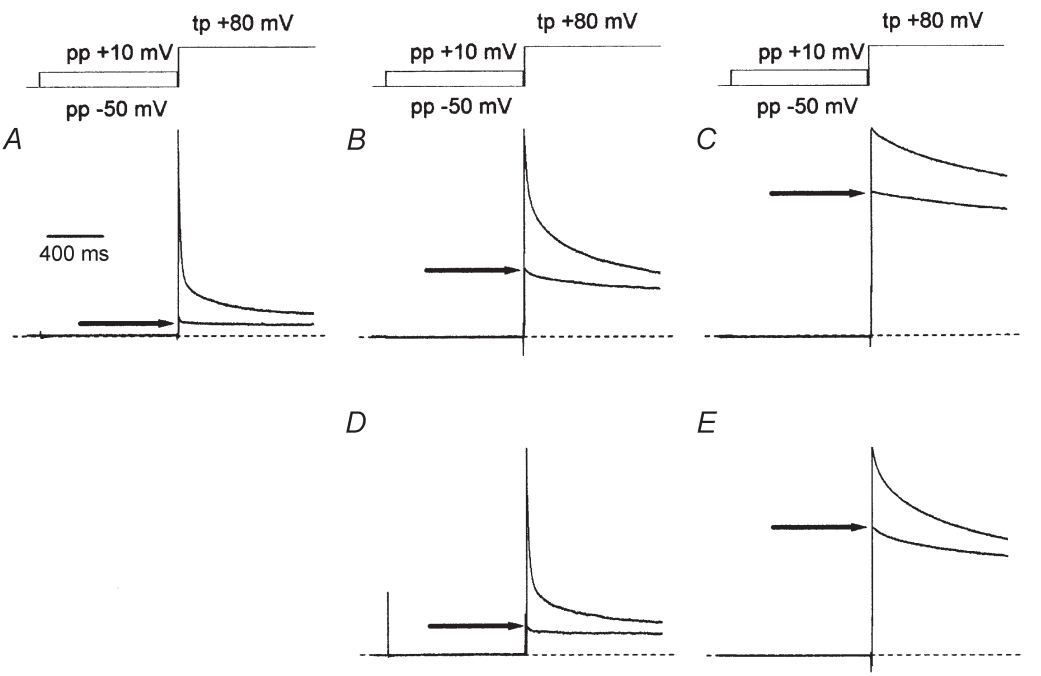

Figure 4. Effects of agonists upon the inactivation of $I_{Ca,L}$ in the absence of extracellular Ca^{2+}

Representative cell currents were recorded in the absence of extracellular Ca^{2+} using the double-pulse voltage-clamp protocol described in Methods. Each of the normalised traces shown are composed of two superimposed current records which were obtained with pre-pulse (pp) voltage steps to either −50 or +10 mV. The voltage-clamp protocol is indicated schematically above the current records. The amplitude of the current evoked by the test pulse (tp) following a pre-pulse to +10 mV is indicated by an arrow in each recording. The dashed lines indicate the zero level. The time scale in *A* applies to all traces. *A*, cell currents recorded under basal conditions. *B*, cell currents recorded in the presence of 2 nM isoproterenol. *C*, cell currents recorded in the presence of 100 nM isoproterenol. *D*, cell currents recorded in the presence of 2 nM isoproterenol and 10 μM carbachol. *E*, cell currents recorded in the presence of 100 nM isoproterenol and 10 μM carbachol. Cell current records shown in *D* and *E* were recorded from the same myocytes as those in *B* and *C*, respectively.

Isoproterenol was also associated with the recovery of channel availability following positive pre-pulse voltage steps, such that the *A–V* relationship that was sigmoid in form under basal conditions was U-shaped.

The effect of carbachol upon the voltage-dependent inactivation of $I_{Ca,L}$ is shown in the cell current records illustrated in Fig. 4*D* and *E*. In the presence of 2 nM isoproterenol (Fig. 4*D*), carbachol clearly enhanced the inactivation of $I_{Ca,L}$ evoked by the test pulse voltage step and increased the inactivation of $I_{Ca,L}$ during the pre-pulse voltage step to +10 mV (arrow in Fig. 4*D*). The addition of 10 μM carbachol to a cell bathed in 100 nM isoproterenol was less effective (Fig. 4*E*). The effects of carbachol upon the *A−V* relationships of $I_{Ca,L}$ stimulated by isoproterenol are shown in Fig. 5. Whether in the presence of 2 nM isoproterenol (●, Fig. 5*A*), 20 nM isoproterenol (●, Fig. 5*B*) or 100 nM isoproterenol (●, Fig. 5*C*), carbachol had no effect upon the voltage dependence of the *A–V* relationships with $V_{0.5}$ values of −19, −21 and −20 mV, respectively. However, carbachol had enhanced the amount of inactivation since the proportions of the total current available for activation at the minima of the *A–V* relationships were reduced to ~20 %, ~40 % and ~50 % in 2, 20 and 100 nM isoproterenol, respectively.

These results strongly suggest that the β-adrenergic and muscarinic agonists influenced the time course of voltage-dependent inactivation of $I_{Ca,L}$ in isolated ventricular myocytes of the guinea-pig. There was no evidence that either agonist influenced the dependence of inactivation upon membrane voltage.

In the absence of extracellular Ca^{2+}, a voltage step to +10 mV did not evoke a measurable current through L-type Ca^{2+} channels (Figs 1*E–H* and 4). Therefore in order to examine the effects of agonists upon the time course of the development of voltage-dependent inactivation of $I_{Ca,L}$ in the absence of Ca^{2+} flux and to compare these data with those for inactivation of $I_{Ca,L}$ due to membrane voltage and Ca^{2+} flux (Fig. 3), it was necessary to measure inactivation indirectly. The time course of the development of voltage-dependent inactivation was therefore assessed by the application of an envelope type of double-pulse voltage-clamp protocol, which measured the availability of channels for activation by a test pulse voltage step to +80 mV, following pre-pulse voltage steps to +10 mV of 10, 20, 50, 100, 200, 500 and 1000 ms duration (Findlay, 2002*b;* and see inset to Fig. 6). Figure 6*A* illustrates the application of this protocol to a myocyte under basal conditions in the absence of external agonists. Although pre-pulse voltage steps to +10 mV of 50, 200 and 1000 ms duration evoked no current flow through L-type Ca^{2+} channels because the cell was bathed in zero calcium extracellular solution, the amplitude of the current evoked by the subsequent voltage step to +80 mV was progressively reduced. This loss of the availability of $I_{Ca,L}$ for activation is considered to reflect the progressive development of inactivation in the channel population during the pre-pulse voltage step. Thus, under basal conditions, voltage-dependent inactivation developed rapidly (Fig. 6*A*) and with a bi-exponential time course (τ_f = 32 ms, τ_s = 380 ms; □, Fig. 6*D*). In the presence of 100 nM isoproterenol, the development of inactivation with time was severely impaired (Fig. 6*B*) and progressed with a single slow exponential time course (τ = 255 ms; ■, Fig. 6*D*). The addition of 10 μM carbachol, in the continued presence of 100 nM isoproterenol, enhanced the development of inactivation (Fig. 6*C*), which again

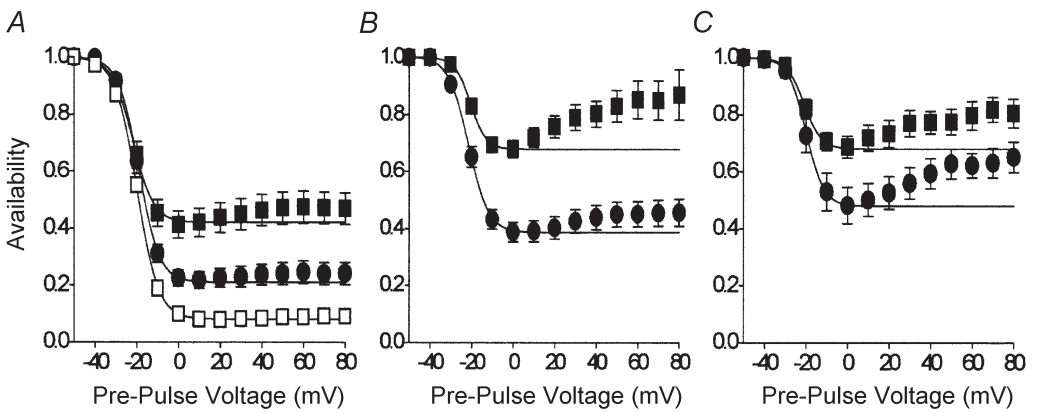

Figure 5. Effects of agonists upon the availability of $I_{Ca,L}$ in the absence of Ca^{2+}

A–V relationships were recorded with the double-pulse voltage-clamp protocol described in Methods. These experiments were conducted with cells bathed in zero calcium extracellular solution. *A*, effects of 2 nM isoproterenol (■, n = 7) and 10 μM carbachol (●). For comparison, data obtained from cells under basal conditions (□, n = 8) are also shown. *B*, effects of 20 nM isoproterenol (■, n = 6) and 10 μM carbachol (●). *C*, effects of 100 nM isoproterenol (■, n = 8) and 10 μM carbachol (●). The lines represent fits of the Boltzmann equation (Origin 4.1) between the maximum and minimum data points, and then extrapolated to +80 mV. See text for further details.

showed a biphasic time course (τ_f = 47 ms, τ_s = 350 ms; ●, Fig. 6*D*).

These results clearly suggest that not only β-adrenergic stimulation but also muscarinic antagonism of the activation of $I_{Ca,L}$ affected the time course of the development of voltage-dependent inactivation. Mitarai *et al.* (2000) and Findlay (2002*b*) both found that, although the time constants of inactivation of $I_{Ca,L}$ were altered by β-adrenergic stimulation, this could be less consequent for $I_{Ca,L}$ as a whole than the redistribution of L-type Ca^{2+} channels between kinetically distinct groups. This was also suggested by analysis of the data shown in Fig. 6*E*, which illustrates the time course of the development of voltage-dependent inactivation recorded in the presence of 2 nM isoproterenol (■) and the effect of the addition of 10 μM carbachol (●). Under basal conditions (□, Fig. 6*D*), inactivation developed with a rapid phase (τ_f = 32 ms), which had an initial amplitude corresponding to ~80 % of the current, and a slow phase (τ_s = 380 ms), which had an initial amplitude of ~15 % of the current. The data recorded in the presence of 2 nM isoproterenol and 10 μM carbachol declined with a very similar time course (τ_f = 28 ms, τ_s = 378 ms; ●, Fig. 6*E*). However, in this case the rapid phase of inactivation corresponded to ~50 % of the current and the slow phase accounted for ~30 % of the current. Overall, therefore, less inactivation of the total current was recorded with time in the presence of 2 nM isoproterenol and 10 μM carbachol (Fig. 6*E*) than under basal conditions (Fig. 6*D*), while the speed of the process of inactivation was the same under both experimental conditions. It was therefore clear that agonists had more than just an effect upon the process of voltage-dependent inactivation (Mitarai *et al.* 2000; Findlay, 2002*b*) and it was decided to examine in further detail the effects of the agonists upon the kinetics of inactivation of $I_{Ca,L}$ recorded in the absence of Ca^{2+}.

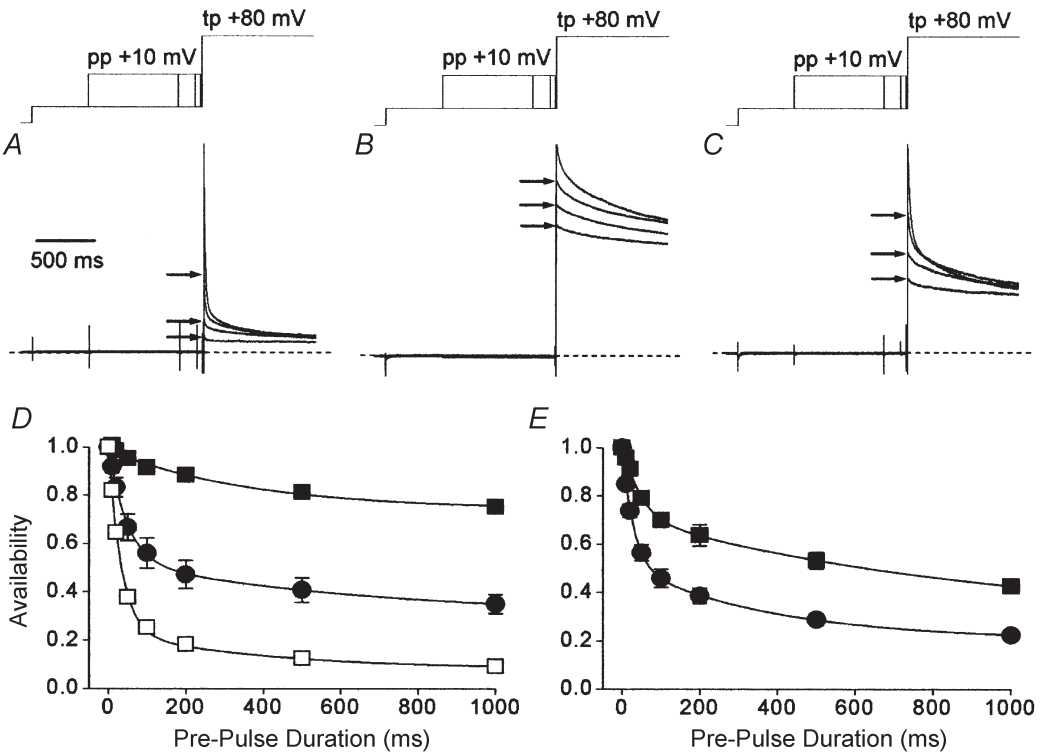

Figure 6. Effects of agonists upon the time course of voltage-dependent inactivation of $I_{Ca,L}$

A, current records obtained from a representative myocyte under basal conditions. *B*, current records obtained from a representative myocyte in the presence of 100 nM isoproterenol. *C*, current records obtained from the cell shown in *B* following the addition of 10 μM carbachol. The voltage-clamp protocol is indicated schematically above the current records. These records each consist of four superimposed normalised traces which were recorded with either no pre-pulse, or pre-pulse voltage steps to +10 mV of 50, 200 and 1000 ms duration, as indicated by arrows. The dashed lines indicate the zero level. The time scale in *A* applies to all three records. *D* and *E*, time course of the development of inactivation of $I_{Ca,L}$. The amplitudes of cell currents evoked by test pulse voltage steps were normalised to those recorded in the absence of a pre-pulse. □ (*D*) represent results obtained from experiments conducted under basal conditions (n = 8). Otherwise, cell currents were recorded first in isoproterenol (■: *D*, 100 nM, n = 6; *E*, 2 nM, n = 6) and then following the addition of 10 μM carbachol (●). The lines represent fits to the data of one or two exponential functions; see text for details.

In this series of experiments, the development of inactivation of $I_{Ca,L}$ was measured directly from the time course of decay of $I_{Ca,L}$ rather than from the indirect loss of availability of $I_{Ca,L}$. The time course of inactivation evaluated from the loss of availability of $I_{Ca,L}$ at +10 mV (τ_f = 32 ms; amplitude, 0.78; τ_s = 380 ms; amplitude, 0.15; offset amplitude, 0.08) was quite similar to the results obtained from the fitting of a double-exponential function (Origin 4.1) to the directly recorded inactivation of $I_{Ca,L}$ at +80 mV ($\tau_f = 15 \pm 1$ ms; amplitude, 0.74 ± 0.06; $\tau_s = 170 \pm 3$ ms; amplitude, 0.16 ± 0.02; offset amplitude, 0.10 ± 0.02; $n = 8$). Therefore, although the data were analysed at what could be considered to be an unphysiological membrane voltage, they closely reflected the physiologically relevant kinetics of inactivation. In addition, it was then also possible to perform an equivalent analysis of the decay of $I_{Ca,L}$ in the presence of Ca^{2+}, where at the same voltage $I_{Ca,L}$ is also outwardly directed (Fig. 1) but not subject to inactivation due to Ca^{2+} influx (see Fig. 9). In each cell the effect of carbachol was tested either under basal conditions or in the presence of a given concentration of isoproterenol. For clarity these data have been separated into two sections. First the dose-dependent effect of the β-adrenergic agonist will be considered (Fig. 7), then the effect of carbachol will be described (Fig. 8).

In a dose-dependent manner, isoproterenol increased the amplitude and reduced the inactivation of $I_{Ca,L}$ recorded in the absence of extracellular Ca^{2+} (Fig. 7*A*). The time course of the development of inactivation could be described by a bi-exponential function. From the bi-exponential function, the components of $I_{Ca,L}$ could be extracted: a component which inactivated rapidly, a component which inactivated slowly, and a time-independent or offset component. Both the fast and slow time constants of inactivation were significantly increased by 2 nM isoproterenol (Fig. 7*B*). Higher concentrations of isoproterenol tended to further increase both time constants, but this increase was not statistically significant. The amplitudes of the distinct kinetic components of $I_{Ca,L}$ were profoundly altered by β-adrenergic stimulation (Fig. 7*C*). Under basal conditions, inactivation of $I_{Ca,L}$ was dominated by a fast time-dependent component. Isoproterenol depressed the amplitude of the fast component in a dose-dependent manner, which was maximal with 20 nM of the agonist (■, Fig. 7*C*). The amplitude of the slow time-dependent component, which was a minor part of the current under basal conditions, was clearly increased by 2 nM

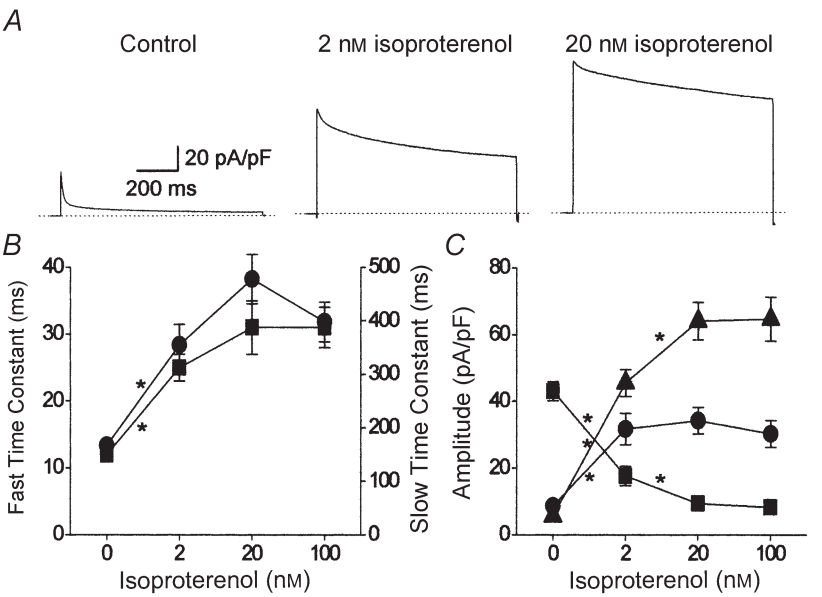

Figure 7. Effect of isoproterenol upon voltage-dependent inactivation of $I_{Ca,L}$

Cells were bathed in zero calcium extracellular solution and currents were evoked by voltage-clamp steps to +80 mV (*A*). The decay of the evoked cell currents was fitted by a bi-exponential function (Origin 4.1) and the components of that function were extracted (*B* and *C*). *A*, representative cell current records obtained from three different myocytes exposed to either basal conditions in the absence of any external agonist (Control; left), 2 nM isoproterenol (centre) or 20 nM isoproterenol (right). The dotted lines indicate the 0 pA current level and the time and current scales shown on the left apply to all three traces. *B*, effect of isoproterenol upon the fast (left axis; ■) and slow (right axis; ●) time constants of decay. Note the different *y*-axis scales for the two time constants. *C*, effect of isoproterenol upon the initial amplitude of fast (■) and slow (●) time-dependent, and the time-independent (▲), components of decay. Control (0 nM isoproterenol), $n = 8$; 2 nM isoproterenol, $n = 7$; 20 nM isoproterenol, $n = 6$; 100 nM isoproterenol, $n = 7$. * $P < 0.05$, for comparison between adjacent groups with one-way ANOVA (Origin 4.1).

isoproterenol, but higher doses of the agonist had no statistically significant further effect (●, Fig. 7*C*). Under basal conditions, time-dependent inactivation of the current was virtually complete after 1000 ms; there remained only a small time-independent portion of the current. Isoproterenol strongly and significantly increased the amplitude of the time-independent current, which reached a maximum with 20 nM of the agonist (▲, Fig. 7*C*). It was therefore clear that the main effect of isoproterenol upon voltage-dependent inactivation of $I_{Ca,L}$ was to alter the amplitudes of separate time-dependent and time-independent kinetic components of the current (Fig. 7*C*). β-Adrenergic stimulation had but little effect upon the actual time course of the process of voltage-dependent inactivation (Fig. 7*B*). It was tempting to speculate that a reciprocal relationship existed between the fast time-dependent (■, Fig. 7*C*) and the time-independent (▲, Fig. 7*C*) kinetic forms of $I_{Ca,L}$.

The effect of carbachol upon the voltage-dependent inactivation of $I_{Ca,L}$ is shown in Fig. 8. It was immediately

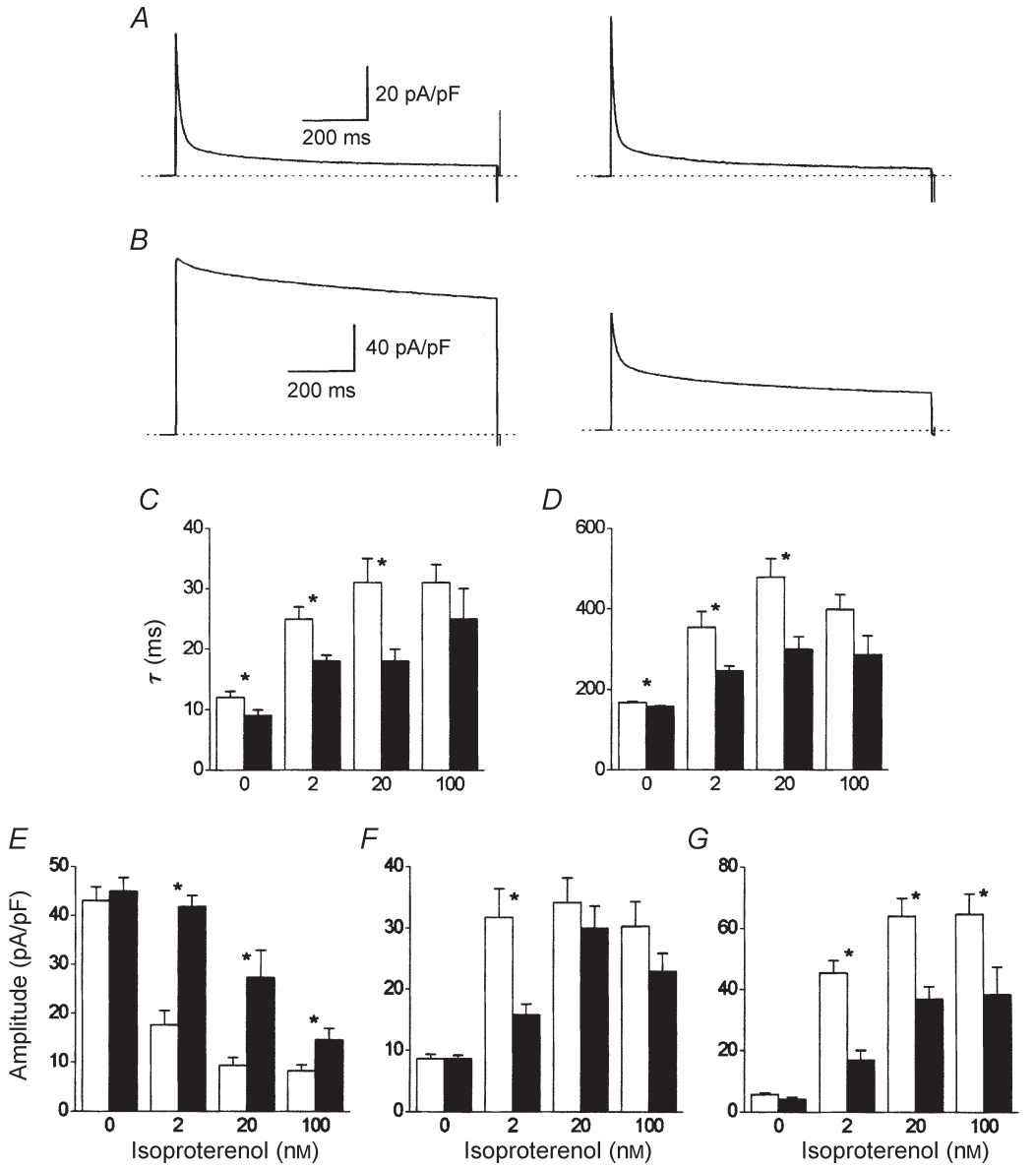

Figure 8. Effect of carbachol upon voltage-dependent inactivation of $I_{Ca,L}$

Experiments and analysis were performed as described in Fig. 7. *A*, effect of 10 μM carbachol (right) upon cell currents recorded in one myocyte under basal conditions (left). *B*, effect of 10 μM carbachol (right) upon cell currents recorded in one myocyte that was bathed in 20 nM isoproterenol (left). *C–G*, analysis of the decay of Ca^{2+} channel currents recorded either under basal conditions or in the presence of isoproterenol (□) and subsequently following the addition of 10 μM carbachol (■). *C*, fast time constants of bi-exponential decay. *D*, slow time constants of bi-exponential decay. *E–G*, initial amplitudes of fast (*E*) and slow (*F*) time-dependent and time-independent (*G*) components of decay. *n* as for Fig. 7*C*. * $P < 0.05$, for the effect of carbachol determined with one-way ANOVA (Origin 4.1).

clear that muscarinic stimulation not only reduced the amplitude of $I_{Ca,L}$ enhanced by isoproterenol but that it also reversed the effects of β-adrenergic stimulation upon the inactivation of $I_{Ca,L}$ (Fig. 8*B*). Under basal conditions carbachol had little effect upon the total amplitude of $I_{Ca,L}$ (Fig. 8*A*), though it significantly accelerated both fast and slow time constants of inactivation (Fig. 8*C* and *D*). Carbachol significantly accelerated the time constants of inactivation of $I_{Ca,L}$ in 2 and 20 nM isoproterenol (Fig. 8*C* and *D*) but not in 100 nM isoproterenol. Carbachol had little significant effect upon the amplitude of the slow time-dependent component of $I_{Ca,L}$, except to reduce the increase that had been induced by 2 nM isoproterenol (Fig. 8*F*). The major effects of carbachol were observed upon the amplitudes of the fast time-dependent (Fig. 8*E*) and the time-independent components of $I_{Ca,L}$ (Fig. 8*G*). Carbachol significantly increased the first and reduced the second at each dose of isoproterenol. The effect of carbachol was 'competitive' with that of isoproterenol, being quantitatively less effective with increasing concentrations of the β-adrenergic agonist. The reciprocal relationship between the amplitudes of the fast time-dependent and the time-independent components of $I_{Ca,L}$ which was first noted with their response to isoproterenol (Fig. 7) was confirmed therefore with their reaction to carbachol (Fig. 8). A large increase in amplitude of the fast time-dependent component of $I_{Ca,L}$ was associated with a large reduction of the time-independent component of $I_{Ca,L}$, and vice versa.

The experiments described in Figs 7 and 8 were conducted upon isolated ventricular myocytes which were bathed in zero calcium extracellular solution. Although previous studies have shown that this is propitious for the isolation of the voltage-dependent process of inactivation of $I_{Ca,L}$ (Hadley & Hume, 1987; Findlay, 2002*b*), it could nevertheless be suggested that the removal of extracellular Ca^{2+} could influence a number of different cellular processes and that its effect would not be confined to the removal of Ca^{2+}-induced inactivation of $I_{Ca,L}$. Figure 9 therefore illustrates analysis of inactivation of $I_{Ca,L}$ evoked by voltage steps to +80 mV in isolated myocytes that were bathed in normal extracellular solution containing 2 mM Ca^{2+}. Analysis of the inactivation of these currents through L-type Ca^{2+} channels with a bi-exponential function extracted fast and slow time-dependent components of $I_{Ca,L}$ and a time-independent component of $I_{Ca,L}$. In a manner similar to the results recorded above in the absence of extracellular Ca^{2+} (Fig. 8), the amplitudes of the fast time-dependent component of $I_{Ca,L}$ (Fig. 9*A*) and the time-independent component of $I_{Ca,L}$ (Fig. 9*C*) were, respectively, decreased and increased by isoproterenol. These effects were counterbalanced by carbachol. These effects of agonist stimulation upon the voltage-dependent inactivation of $I_{Ca,L}$ therefore do not reside in a non-specific effect of withdrawal of extracellular Ca^{2+} upon the physiology of ventricular myocytes.

DISCUSSION

Voltage-dependent inactivation of cardiac muscle $I_{Ca,L}$ determines not only the kinetics of decay of Ca^{2+} currents in the heart but also the extent to which Ca^{2+}-induced inactivation can contribute to this process (Findlay, 2002*a*,*b*). In this study, evidence is presented which suggests that modulation of voltage-dependent inactivation of $I_{Ca,L}$ by sympathetic and parasympathetic agonists is quite simple. Phosphorylation of the channel by the action of PKA prevents the operation of the mechanism of voltage-dependent inactivation. This can be considered to represent an 'off' switch. Under basal conditions, the application of a voltage step provokes the channel molecule to adopt first a

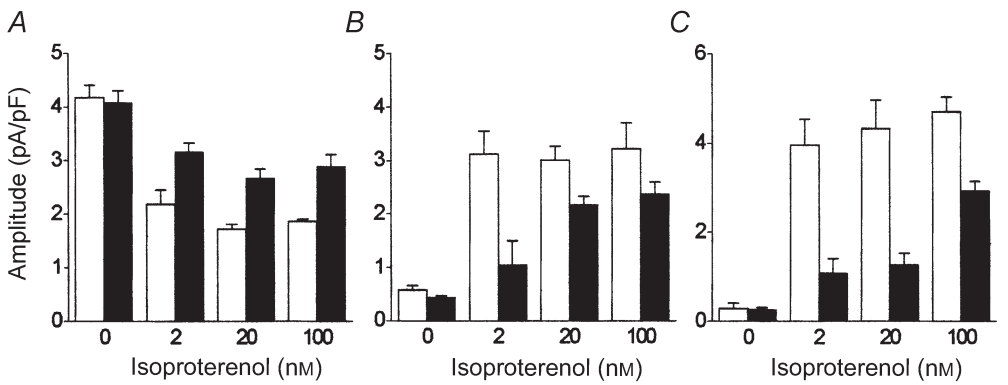

Figure 9. Effects of agonists upon voltage-dependent inactivation of $I_{Ca,L}$ in the presence of extracellular Ca^{2+}

Experiments and analysis were performed as described in Fig. 7 on cells bathed in normal extracellular solution containing 2 mM Ca^{2+}. Cell currents evoked by a voltage step to +80 mV were recorded either under basal conditions (0 nM) or in the presence of isoproterenol (□) and subsequently following the addition of 10 μM carbachol (■). The initial amplitudes of fast (*A*) and slow (*B*) time-dependent and time-independent (*C*) components of decay are shown. *n* as for Fig. 3*B*.

configuration that allows the opening of the channel pore and then a configuration where ion passage is occluded. The action of PKA would be to prevent the assumption of the inactivated configuration by the channel. In consequence, during a sustained voltage step, openings and closings of the ion channel would continue. There would no longer be a time-dependent decay of the current.

It was not the objective of this study to determine the intracellular signalling pathways by which β-adrenergic and m_2-muscarinic agonists affect their targets. This has been extensively investigated and described in recent reviews by Méry *et al.* (1997) and Kamp & Hell (2000). It is agreed that neither agonist directly affects $I_{Ca,L}$ and that both agonist systems converge upon the modulation of PKA. The effects of carbachol described here therefore correspond to reduction of the activation of PKA and reduction of the phosphorylation of the Ca^{2+} channel protein(s). The idea therefore that the β-adrenergic dose-dependent redistribution of kinetic behaviour in the ion channel population results from ion channel phosphorylation due to the action of PKA is reinforced by results showing that carbachol reverses the kinetic behaviour of the channels and re-establishes time-dependent decay. The process of voltage-dependent inactivation has been turned on.

There is some evidence that agonist stimulation influenced the time constants of decay of fast and slow components of the channel population (Figs 7*B* and 8*C* and *D*). This might suggest a graded response of the L-type Ca^{2+} channel to agonist stimulation. These effects were significant at low doses of the β-adrenergic agonist, not at high doses. However, Mitarai *et al.* (2000) and a previous study in this laboratory (Findlay, 2002*b*) both showed that changes in the time course of decay were of little consequence compared with the very large difference in currents resulting from the redistribution of ion channels between the different kinetic groups. It would be interesting to investigate, using single-channel current recording methods, the kinetic behaviour characterising channels under basal conditions and that seen following agonist stimulation. These experiments would have to be conducted in the absence of divalent cations in order to avoid complications resulting from localised ion-dependent inactivation (Findlay, 2002*a*).

Voltage-dependent inactivation of Ca^{2+} channels has been the subject of extensive investigation at the molecular level (see Stotz & Zamponi, 2001, for a recent review). The objectives of that work were to investigate the mechanism and the molecular attributes necessary for the rapid time-dependent decay shown by certain Ca^{2+} channels. This involved the mix-and-match method of interchanging segments from Ca^{2+} channel molecules exhibiting different forms of inactivation. Alternatively, the role of accessory subunits of the Ca^{2+} channel molecular complex has been suggested to determine the behaviour of the central pore-forming α_1 protein (Birnbaumer *et al.* 1998; Bers & Perez-Reyes, 1999). The message from the results described here, and from previous studies (Mitarai *et al.* 2000; Findlay, 2002*b*), is that voltage-dependent inactivation of the L-type Ca^{2+} channel current of cardiac muscle is not a fixed characteristic. Rather that either rapid or no time-dependent decay of the current are characteristics of the same molecular complex, for which an explanation at the molecular level remains to be determined. Some recent studies have suggested that interactions between different subunits of the Ca^{2+} channel might be subject to modulation by physiological processes (Restituito *et al.* 2001). It may be that this line of research may lead to the elucidation of the mechanism of the turning on and off of voltage-dependent inactivation of the L-type Ca^{2+} channel of cardiac muscle.

Two aspects of the experimental approach used in this investigation could be considered unusual in the context of an examination of the behaviour of a native Ca^{2+} channel current. First, the behaviour of the channel was evaluated from the outward flux of monovalent cations and not from the inward flux of divalent cations. Second, in part the kinetics of inactivation of the current were evaluated at +80 mV, which could be considered not to represent a physiologically relevant membrane potential. Previous studies (Hadley & Hume, 1987; Hadley & Lederer, 1991; Findlay, 2002*b*) have shown that the replacement of extracellular Ca^{2+} by Mg^{2+} and the monitoring of $I_{Ca,L}$ carried by the outward flux of intracellular monovalent cations isolated the process of voltage-dependent inactivation of $I_{Ca,L}$ (see also Fig. 5). It is admitted that the currents carried by L-type Ca^{2+} channels under these circumstances (Fig. 1) have the appearance of a transient outward K^+-selective ion conductance, and Inoue & Imanaga (1993) described them as an A-type K^+ current that was inhibited by the presence of extracellular Ca^{2+}. However, these currents are not K^+ selective (Bean *et al.* 1984), cannot be blocked by blockers of transient outward currents (Inoue & Imanaga, 1993; Findlay, 2002*b*), but can be blocked by blockers of L-type Ca^{2+} currents (Hadley & Hume, 1987; Inoue & Imanaga, 1993; Findlay, 2002*b*). They result from the asymmetric permeation of cations through Ca^{2+} channels (Tsien *et al.* 1986). The advantage of this method of recording $I_{Ca,L}$ is twofold. First, ion-dependent inactivation of the current is avoided; this would usually result from Ca^{2+} influx but can also be evoked by Ba^{2+} and Sr^{2+} (Ferreira *et al.* 1997; Findlay, 2002*a*). Second, disruption of membrane surface charge and the transmembrane voltage field arising from the chelation of extracellular divalent cations to allow monovalent cation influx through L-type Ca^{2+} channels (Fukushima & Hagiwara, 1985; Mitarai *et al.* 2000; Findlay, 2002*a*) is countered by the presence of the impermeant Mg^{2+} (Hadley & Hume, 1987; Findlay,

2002*b*). The kinetics of decay of $I_{Ca,L}$ were evaluated indirectly (Fig. 6) and directly (Figs 7–9). The former method involved use of the amplitude of the current evoked by a voltage step to +80 mV as a measure of the availability of the ion channel (Hadley & Hume, 1987; Hadley & Lederer, 1991; Findlay, 2002*b*), in a manner that was no different from the use of double-pulse voltage-clamp protocols involving test pulse voltage steps to 0 or +10 mV, with equivalent results (Findlay, 2002*b*). The latter method also involved use of the currents evoked by voltage steps to +80 mV as a measure of the process of inactivation. There was little difference between the kinetics of decay obtained using the direct method and those evaluated to occur at +10 mV. Separate experiments (not shown) have shown that the kinetics of voltage-dependent inactivation increase from −30 to ~+20 mV and that further depolarisation has no further effect. The decay kinetics recorded at +80 mV therefore adequately report the maximal and physiologically relevant time course of inactivation.

The effects of the opposing agonists isoproterenol and carbachol upon $I_{Ca,L}$ carried by Ca^{2+} obtained in this investigation (Figs 1 and 2) were very similar to those observed in previous reports involving native cardiac Ca^{2+} currents. The unusual nature of their effects upon voltage-dependent inactivation therefore does not arise from any particular characteristic of the experiments performed here. Thus β-adrenergic stimulation evoked negative shifts of the peak of the *I–V* relationship and *A–V* curves (McDonald *et al.* 1994). The effects of carbachol were 'competitive' with those of isoproterenol (Fischmeister & Shrier, 1989). On the other hand, the observation that neither agonist directly influenced the dependence of inactivation upon membrane voltage recorded in the absence of Ca^{2+} is new (Fig. 5). This suggests that the shift of the relationship between total inactivation and membrane voltage effected by agonist stimulation (Fig. 2) resulted from enhanced Ca^{2+} influx and enhanced Ca^{2+}-induced inactivation.

The physiological importance of the ensemble of the results obtained in this investigation lies in the observation that, when Ca^{2+} carried the Ca^{2+} current, the effects of the β-adrenergic and muscarinic agonists upon the voltage-dependent decay of the current were not observed (Fig. 3). Their modification of the behaviour of the ion channel had been over-ridden by the influence of Ca^{2+}-induced inactivation (Findlay, 2002*b*). This therefore identifies quite clearly the manner by which the alteration of the behaviour of the channel molecule could be compensated for by its environment. Any mechanism or pathology interfering with the process of Ca^{2+}-induced inactivation will therefore have consequences that will depend upon the demands placed upon the heart.

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Acknowledgements
I thank Joel Derisson for isolating cells and Dr Claire Malecot for her constructive criticism of this text. This work was financed by grants from the Region Centre.