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Ca^{2+} CHANNEL MODULATING EFFECTS OF HEPARIN IN MAMMALIAN CARDIAC MYOCYTES

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SUMMARY

1. The effect of heparin on L-type Ca^{2+} channels in rabbit, rat and guinea-pig cardiac myocytes was studied using the whole-cell patch clamp method.

2. Sodium salts of heparin uniformly suppressed the Ca^{2+} current, I_{Ca}, independent of their molecular weight, in the rat and guinea-pig ventricular and rabbit atrial myocytes. The suppression of I_{Ca} by heparin was dose dependent and reached its maximum, about 30%, around 10 μM. Heparin did not alter the voltage-dependence or the steady-state inactivation properties of I_{Ca}. These effects were specific to heparin as another polysaccharide, dextran, failed to have any effect on I_{Ca}.

3. The suppressive effect of heparin was not diminished when $[Ca^{2+}]_o$ was increased to 10 mM, or when Ba^{2+} was the charge carrier through the Ca^{2+} channel.

4. Spectrophotometric assays showed that heparin-induced changes in $[Ca^{2+}]_o$ generally were too small to alter I_{Ca} significantly.

5. In myocytes buffered with 0·1 mM EGTA, the suppressive effect of heparin was more prominent on the inactivating than on the maintained component of I_{Ca}.

6. When extracellular Na^+ was replaced by Cs^+, the heparin suppressive effect was accompanied by a 10 mV shift of both the voltage dependence of activation and the steady-state inactivation parameters toward more negative potentials.

7. When both Mg^{2+} and Na^+ were omitted from the bathing solutions, the suppressive effect of heparin was significantly enhanced such that almost 80% of the current was blocked.

8. In Cs^+-based solutions 10 mM $[Mg^{2+}]_o$ suppressed I_{Ca} by about 70% and heparin partially relieved this block. Heparin, however, did not counteract the Mg^{2+}-induced suppression of I_{Ca} in Na^+-based solution.

9. Extracellularly applied heparin did not alter the isoprenaline-induced enhancement of I_{Ca} or interfere with the blocking effect of phorbol esters on I_{Ca}.

10. Heparin thus appears to interfere with the permeation of Ca^{2+} through the channel by a mechanism regulating the Ca^{2+}-induced inactivation of the Ca^{2+} channel. Na^+ and Mg^{2+} appear to alter the kinetics and the magnitude of the

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suppressive effect of heparin on the Ca^{2+} channel, suggesting an interaction of these cations with either the Ca^{2+} or the heparin-binding sites of the channel.

INTRODUCTION

Heparin is a naturally occurring mucopolysaccharide with the molecular weight ranging from 3000 to 40000. It is produced in the lungs, spleen and liver with the human plasma concentration level at about 1·5 μg/ml. The fundamental units of this polymer are thought to be hexa- or octasaccharides with molecular weight of 1500 or 2000 (Silva & Dietrich, 1975).

In addition to the well studied role of heparin in coagulation and lipid metabolism, heparin appears also to be involved in cell-to-cell communication, and membrane transport processes. Heparin, for instance, inhibits the Na^+–H^+ exchange in vascular smooth muscle cells (Zaragoza, Battle-Tracy & Owen, 1990), by inhibiting serum- or growth factor-stimulated calcium efflux from the intracellular compartment. Heparin decreases the activity of adenylate cyclase stimulated by various agonists like prostaglandin E_1 (Reches, Eldor & Salomon, 1979) and isoprenaline (Cutler & Christian, 1984), inhibits the inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from the sarcoplasmic reticulum (Worley, Baraban, Supattapone, Wilson & Snyder, 1987; Kobayashi, Kitazawa, Somlyo & Somlyo, 1989), stimulates calcium uptake in canine cardiac microsomes (Xu & Kirchberger, 1989), uncouples α_2-adrenoceptors from the G_i protein in membranes of human platelets (Willuweit & Aktories, 1988), uncouples the muscarinic receptors from G_k protein in the atrial cell membrane of the guinea-pig heart (Ito, Takikawa, Iguchi, Hamada, Sugimoto & Kurachi, 1990), and prevents agonist-dependent phosphorylation of the β-adrenergic and muscarinic receptors (Lohse, Lefkowitz, Caron & Benovic, 1989; Kwatra, Benovic, Caron, Lefkowitz & Hosey, 1989). All these effects are caused by direct interaction of heparin with the internal side of the cell membrane, and would require a specialized transport system as has been described already in vascular myocytes (Castellot, Addonizio, Rosenberg & Karnovsky, 1981).

Heparin may also modulate cell membrane proteins such as the Ca^{2+} channels by directly binding to the putative extracellular domain connecting the fifth and sixth putative transmembrane segment in the α_1 subunit of skeletal muscle L-type Ca^{2+} channel (Knaus, Scheffauer, Romanin, Schindler & Glossmann, 1990). This binding appears to inhibit non-competitively the phenylalkylamine, benzothiazepine and 1,4-dihydropyridine binding sites of L-type Ca^{2+} channel (Knaus *et al.* 1990). Since heparin was reported to enhance Ca^{2+} current (Knaus *et al.* 1990), we examined in greater detail the effect of various heparins on the Ca^{2+} currents of rat, guinea-pig and rabbit ventricular and atrial myocytes in an attempt to gain some insight into its mechanism of action. Consistent with biochemical studies of Knaus *et al.* 1990, we found that heparins rapidly and directly interact with the Ca^{2+} channel, but in contrast to these studies, we found only an inhibitory effect on I_{Ca} with either high or low molecular weight heparin, irrespective of species of myocyte and the source of heparin used. A preliminary report of these studies has already appeared (Lacinova & Morad, 1991).

METHODS

Preparation and cell isolation

Myocytes were enzymatically isolated from hearts of deeply anaesthetized (sodium pentabitone 60–100 mg/kg) rats, guinea-pigs, and rabbits (Mitra & Morad, 1985). Membrane currents were recorded under whole-cell clamp conditions (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using a Dagan amplifier series 8900. Patch electrodes had resistances of 1·5–2·5 MΩ and were fire-polished. A concentration-clamp system (Vibraspec Inc., Bear Island, ME, USA) was used to exchange the extracellular solution around individual cells in less than 100 ms.

The measured currents were normalized relative to the membrane capacitance of individual cells (C_m = 50–200 pF) and are expressed in units of pA/pF. Current–voltage (I–V) relations were measured with depolarizing voltage-clamp pulses of 30 ms duration and varying amplitude applied at 5 s intervals from a fixed holding potential (V_h). The effect of heparin was tested only after stable recordings of I_{Ca}, with little or no indication of run-down, for at least 3–5 min. Steady-state inactivation curves, or availability curves, were measured by plotting the peak inward current recorded during a 30 ms test pulse to 0 mV *versus* the variable potential imposed during preceding conditioning clamp pulses of 500 ms duration. Inactivation curves are shown both scaled to unity at their most negative potential (−70 to −100 mV) and, in insets, as the unscaled currents (panel *C* of Figs 2, 7, 8 and 9).

Solutions

The pipette solution used for intracellular dialysis varied according to experimental requirement but generally contained (mM): 115 CsCl, 5 NaCl, 5 MgATP, 20 TEACl, 0·1 or 10 EGTA, 0·2 GTP, 0–0·05 cAMP, and 20 Hepes buffer at pH 7·2. The standard extracellular solution contained (mM): 137 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 0–0·1 heparin, and 10 Hepes at pH 7·4 and 20–24 °C. In some experiments the extracellular concentration of $MgCl_2$ was varied from 0 to 10 mM and/or NaCl was replaced by CsCl (Table 1) in order to investigate the possible influence of extracellular Na^+ and Mg^{2+} on the heparin-induced suppression of I_{Ca}. In other experiments the extracellular Ca^{2+} concentration of the standard solution was increased from 2 to 10 mM or $CaCl_2$ was isotonically replaced with $BaCl_2$ while 0·1 mM EGTA was added to bind residual Ca^{2+} contaminants (Katzka & Morad, 1991). K^+ was omitted from all solutions to block the K^+ currents. Frequently 5 μM tetrodotoxin (TTX) was used to suppress the Na^+ current.

Heparins

Four types of heparin were used: (1) high molecular weight heparin, sodium salt (MW ≃ 15000–18000, Sigma H-3125; 176 USP units/mg), referred to here as 'HMW heparin'; (2) heparin–sodium solution for clinical use (MW ≃ 40000; Elkins-Sinn Inc. Cherry Hill, NJ, USA; 1000 units/ml), referred to here as 'clinical heparin'; (3) low molecular weight sodium salts of porcine intestinal mucosa heparin (MW ≃ 4000–6000, Sigma H-5640; > 140 units/mg) or of bovine intestinal mucosa heparin (MW ≃ 3000, Sigma H-9518), both referred to here as 'LMW heparin'; and (4) calcium salt of heparin from porcine intestine (Fluka 51542; 110 USP units/mg), referred to here as 'Ca-heparin'. Since there was a range of heparins with different molecular weights even in LMW and HMW heparin, we used an average molecular weight within the range of molecular weights given to estimate the approximate concentration of heparin in solution. For instance, the concentration of 100 μg/ml HMW heparin used in many experiments is estimated to be equivalent to 6 μM based on an average molecular weight of 16500. The pH of solutions was always remeasured, and, if necessary, corrected following addition of heparin.

Spectrophotometric assays of $[Ca^{2+}]_o$ *and* $[Mg^{2+}]_o$

Metallochromic dyes were used to test if the binding of cations to heparin significantly altered the concentration of Ca^{2+} and Mg^{2+} in the perfusing solutions. The Ca^{2+} concentration was measured with 50 μM tetramethyl murexide (TMX; Onishi, 1978) by differential absorbance measurements (1 cm cuvette, LKB Ultraspec II). The addition of Ca^{2+} to a previously Ca^{2+}-free solution increased the absorption at a wavelength of 500 nm and decreased it at 545 nm, on the other side of the isosbestic point. A calibration curve was obtained by plotting the difference between the two absorbances *versus* the value of $[Ca^{2+}]_o$ calculated from the amount of added Ca^{2+}. The calibration curve was then used to determine changes in $[Ca^{2+}]_o$ produced by heparin. The Mg^{2+}

concentration in Ca^{2+}-free solutions was determined with 20 μM mag-fura-2 (Molecular Probes, Eugene, OR, USA) from the absorbances at 325 and 375 nm.

RESULTS

Binding of Ca^{2+} to heparin

Heparins are sulphated polyanionic oligosaccharides. Each disaccharide moiety carries about four negative charges (Choay, 1989) and contributes, as Na^+ or Ca^{2+} salt, about 660 to the molecular weight, not counting hydration by as many as sixteen water molecules (Grant, Long & Williamson, 1990). It is possible, therefore,

TABLE 1. Composition of solutions

	A	B	C	D	E	F
NaCl (mM)	137	137	137	0	0	0
CsCl (mM)	0	0	0	137	137	137
$MgCl_2$ (mM)	0	1	10	0	1	10
HMW heparin Ca^{2+} binding (μmol/mg)	0·68 ±0·05	0·61 ±0·04	0·52 ±0·04	0·70 ±0·07	0·67 ±0·07	0·62 ±0·07
Ca-heparin Ca^{2+} release (μmol/mg)	2·00 ±0·03	2·07 ±0·02	2·10 ±0·02	2·04 ±0·02	2·11 ±0·02	2·18 ±0·01
Current amplitude (%)	−16·2 ±1·8	−29·3 ±3·5	No change	−77·9 ±4·0	−41·6 ±1·5	+53·0 ±10
Shift in peak of *I–V* (mV)	None	None	None	−10	−5	−10
Shift in inactivation curve (mV)	None	None	None	−10	−10	−10

Heparin as Ca^{2+} chelator (middle) and inhibitor of I_{Ca} (bottom) in different solutions (top). Solution B is the standard solution on which other modifications are based. The accuracy of the measurements of Ca^{2+} binding is related mainly to the consistency of the spectro-photometric measurements. Changes in I_{Ca} in rat ventricular myocytes were determined as the average value (±S.E.M.) of the reductions measured in individual cells. Shifts in *I–V* relations were determined to the nearest 5 mV from three or more experiments.

that two Ca^{2+} ions could bind to each dissacharide moity (660) corresponding approximately to 1 mg (2/660) = 3 μmol of Ca^{2+} per milligram of heparin. In an experiment where 100 μg heparin salt was added per millilitre of solution the Ca^{2+} concentration of the solution could then change by as much as 300 μM ((3 μmol/mg) (100 μg/ml)) causing a significant departure from the nominal Ca^{2+} concentration of the solution (2 mM). This possibility was tested with the Ca^{2+} indicator dye tetramethyl murexide. Addition of HMW heparin, keeping the total concentration of bound and free Ca^{2+} constant at 2 mM, gave absorption changes indicative of a non-linear reduction of $[Ca^{2+}]_o$ (Fig. 1*A*). The Ca^{2+} binding of small concentrations of HMW heparin was determined from the initial slope of a smooth curve approximating the measured points (Fig. 1*A*, Table 1). The amount of Ca^{2+} bound by heparin was not significantly different in Na^+- and Cs^+-based solutions but was reduced in solutions where the Mg^{2+} concentration was elevated. In a solution with 100 μg/ml HMW heparin it is estimated that $[Ca^{2+}]_o$ is reduced by 80 μM or less.

The effect of the Ca^{2+} salt of heparin was quite different from that of the Na^+ salt

of HMW heparin since its addition changed the TMX absorbance in a manner which mimicked the addition of $CaCl_2$ (Fig. 1*B*, Table 1). The amount of Ca^{2+} released from Ca-heparin was augmented in the presence of Mg^{2+} supporting the idea that heparin binds less Ca^{2+} in Mg^{2+}-rich solutions. Once again, there was no significant difference between the Na^+- and Cs^+-based solutions. The addition of 100 μg/ml Ca-heparin was estimated to be sufficient to elevate $[Ca^{2+}]_o$ from 2 to 2·21 mM.

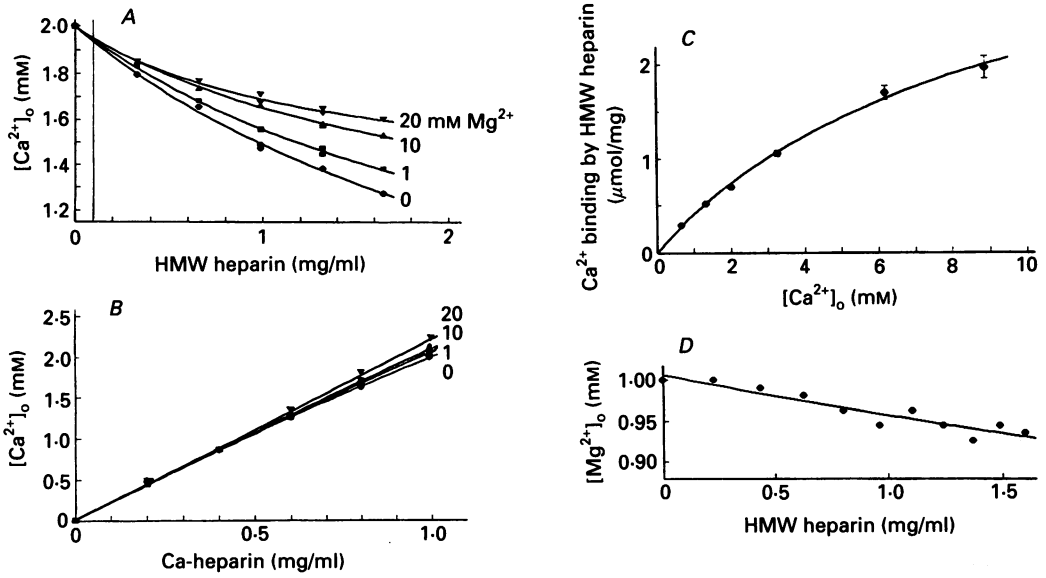

Fig. 1. Effects of heparin on $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$. Panel *A* shows the suppression of $[Ca^{2+}]_o$ produced by addition of the Na^+-salt of HMW heparin. This suppression was reduced by elevation of $[Mg^{2+}]_o$ concentration from 0 (●, solution A) to 1 (■, solution B), 10 (▲, solution C) and 20 (▼) mM. The points for each value of $[Mg^{2+}]_o$ were approximated by a hyperbola by least squares regression and the slope corresponding to small concentrations of heparin was entered into Table 1 HMW heparin binding column A–C. The horizontal line at 100 μg/ml corresponds to the heparin concentration used in most experiments. Panel *B* shows the elevation of $[Ca^{2+}]_o$ produced by the addition of Ca-heparin to previously Ca^{2+}-free solutions containing from 0 to 20 mM Mg^{2+}. The values listed in Table 1 are determined from the concentration of heparin which raised $[Ca^{2+}]_o$ to 2 mM. Panel *C* shows that the binding of Ca^{2+} to HMW heparin increases as $[Ca^{2+}]_o$ is increased in the range from 0·6 to 10 mM. The measurements were performed with Na^+-based Tyrode solution without Mg^{2+} and each point is based on a titration curve of the type shown in panel *A*. Thus, the lower curve in panel *A* shows that addition of 1 mg/ml HMW heparin lowers $[Ca^{2+}]_o$ from an initial value of 2 mM by 0·52 mM to 1·48 mM and therefore gives values (1·48 mM, 0·52 μmol/mg) corresponding, approximately, to the second point on the curve in panel *C*. The error bars in panel *C* are calculated from the repeatability of the spectrophotometric measurements estimated to correspond to 0·0025 absorbance units. Panel *D* shows the slight suppression of $[Mg^{2+}]_o$ produced by addition of HMW heparin to a Na^+-based Tyrode solution with 1 mM Mg^{2+} but no Ca^{2+}. Separate calibration curves were used for each titration to minimize second order effects of Cs^+ and Na^+ on both TMX and mag-fura-2 and of Mg^{2+} on TMX.

In solution with 2 mM Ca^{2+}, the amount of Ca^{2+} released from Ca-heparin (2–2·2 μmol/mg, Table 1) was substantially larger than the amount bound by HMW heparin (0·5–0·7 μmol/mg). This might indicate that higher concentrations of Ca^{2+} are required to yield more than 50% saturation of the binding sites of heparin. To test this possibility we repeated the experiments of the type shown in Fig. 1*A*, with different initial Ca^{2+} concentrations and found (Fig. 1*C*) that the amount of Ca^{2+} bound to HMW heparin increased to 2 μmol/mg or more as Ca^{2+} was elevated to about 10 mM. The detailed saturation kinetics were not accurately determined as the useful range of TMX as a Ca^{2+} indicator dye (dissociation constant (K_d) = 3·6 mM) was exhausted (see error bars) prior to saturation of HMW heparin.

Mag-fura-2 was used to measure the effect of HMW heparin on $[Mg^{2+}]_o$. In solutions containing 100 μM EGTA and no added Ca^{2+}, it was estimated that addition of 100 μg/ml HMW heparin lowered $[Mg^{2+}]_o$ from a control value of 1 mM by only 5 ± 3 μM in Na^+-based Tyrode solution (Fig. 1*D*) and 8 ± 3 μM in Cs^+-based Tyrode solution.

These measurements are consistent with the idea that heparin binds divalent cations competitively with a total binding capacity of about 2·7 μmol/mg and with K_ds of about 6 mM for Ca^{2+} and 20 mM for Mg^{2+}. Such a unified description serves to obtain rough estimates of the changes in $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ produced by addition of heparin. The measurements, however, did not extend to the region where saturating binding is approached (Scott, 1973) nor do they preclude the existence of smaller populations of binding sites with different selectivity or affinity.

Heparin suppresses calcium current

Figure 2 shows that heparin suppressed calcium current without altering its kinetics. The suppressive effect had a rapid onset and was complete in 80–200 s, depending on the type of myocyte and heparin used. The effect of long exposures of heparin was only partially reversible. Shorter exposures (30–50 s) were often easier to reverse. The initial rapid response (5–10 s) to heparin, and the recovery of I_{Ca} following brief exposure to heparin indicate that run-down of I_{Ca} does not invalidate the results. The voltage dependence of I_{Ca} was not significantly altered even though peak I_{Ca} was suppressed (Fig. 2*B*). The steady-state availability of the Ca^{2+} current was also not significantly altered by heparin (Fig. 2*C*). These effects were found consistently in twenty-three rat and ten guinea-pig cardiac myocytes under control conditions (solution B, Table 1).

The suppressive effect of heparin in rat and guinea-pig ventricular myocytes under control conditions (solution B) is summarized in scattergrams (Fig. 3*A* and *B*). Since the density of I_{Ca} under control conditions varied greatly from cell to cell (partly because the concentration of cAMP varied from 0 to 50 μM) we chose to represent the suppressive effect of heparin as the deviation from the unity line. Points below this line indicate suppression of I_{Ca}. In no case did heparin increase I_{Ca}. I_{Ca} was uniformly suppressed by HMW heparin, clinical heparin and LMW heparin. In rat ventricular myocytes the degree of suppression was roughly the same whether the normalized current was small (6–15 pA/pF) or large (15–28 pA/pF). Ca-heparin, on the other hand, produced no detectable suppression of I_{Ca} when applied at a concentration of

100 μg/ml, possibly because the effect of heparin was masked by the elevation of $[Ca^{2+}]_o$. This idea is supported by a rapid (< 5 s) and reversible elevation in I_{Ca} observed when a 10 times higher concentration of Ca-heparin was used such as to increase the Ca^{2+} content of the perfusate by about 2 mM (see Fig. 1*B*). To avoid such

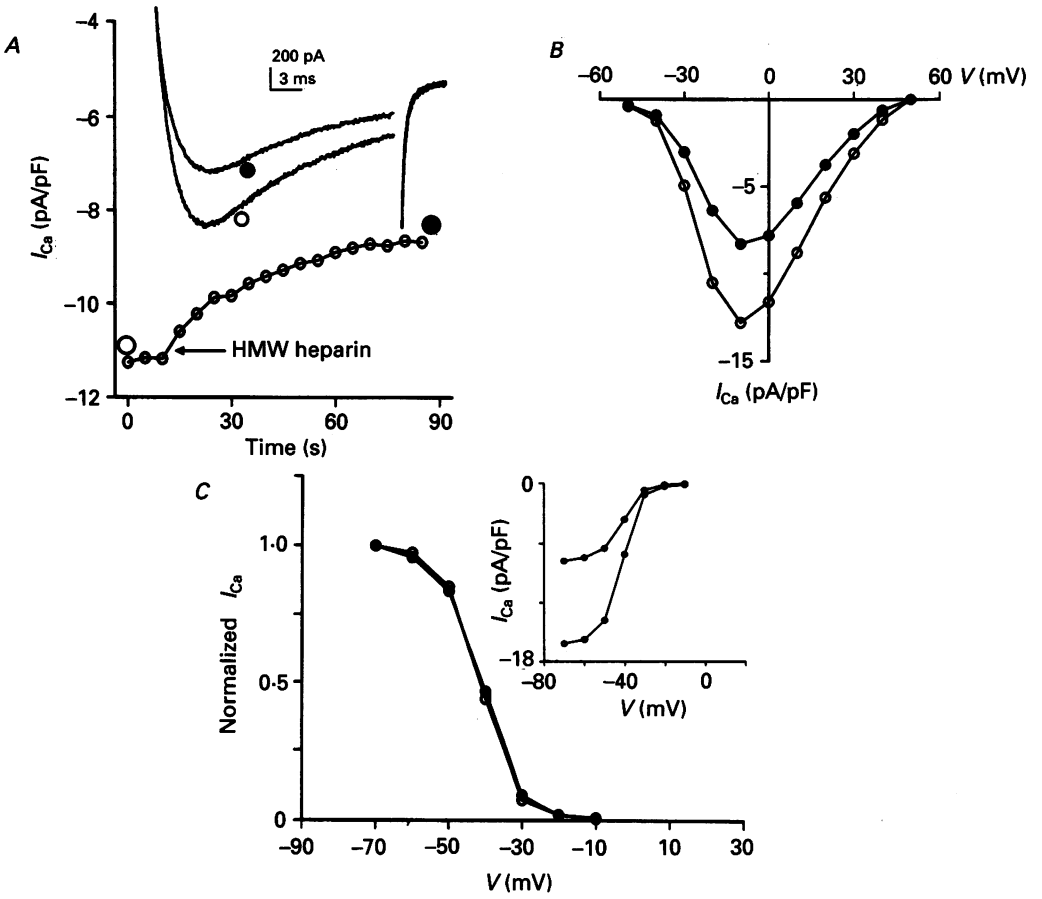

Fig. 2. Heparin suppresses the calcium current. Panel *A* shows the time course of the effect of 100 μg/ml HMW heparin on I_{Ca}. I_{Ca} was activated by 30 ms depolarizing pulses to −10 mV every 5 s (holding potential, $V_h = -60$ mV). Calcium current traces in the absence (○) and presence (●) of heparin are shown in the inset. Panel *B*, current–voltage (*I–V*) relationship measured in the absence (○) and presence (●) of heparin ($V_h = -60$ mV). Panel *C*, steady-state inactivation of I_{Ca} in the absence (○) and presence (●) of heparin. I_{Ca} was activated by 30 pulses to 0 mV immediately following 500 ms conditioning pulses to potentials between −70 and 0 mV. The inactivation curves scaled to unity at −70 mV show that heparin did not alter the voltage dependence of the inactivation process. Currents in panels *A* and *B*, and in the inset of panel *C* were normalized with respect to the cell capacitance, $C_m = 104$ pF.

complications the experiments described below were all performed with the Ca^{2+}-free heparin compounds.

The suppressive effect of heparin on I_{Ca} did not depend on the intracellular cAMP concentration. In the experiments described below, we used 10 μM cAMP in the

pipette solution to slow down the run-down of I_{Ca} and maintain a current density of 10–20 pA/pF. This concentration of cAMP also shifted the steady-state inactivation of I_{Na} toward more negative potentials making it possible to measure I_{Ca} at holding potentials (V_h) of −60 mV even in the absence of TTX.

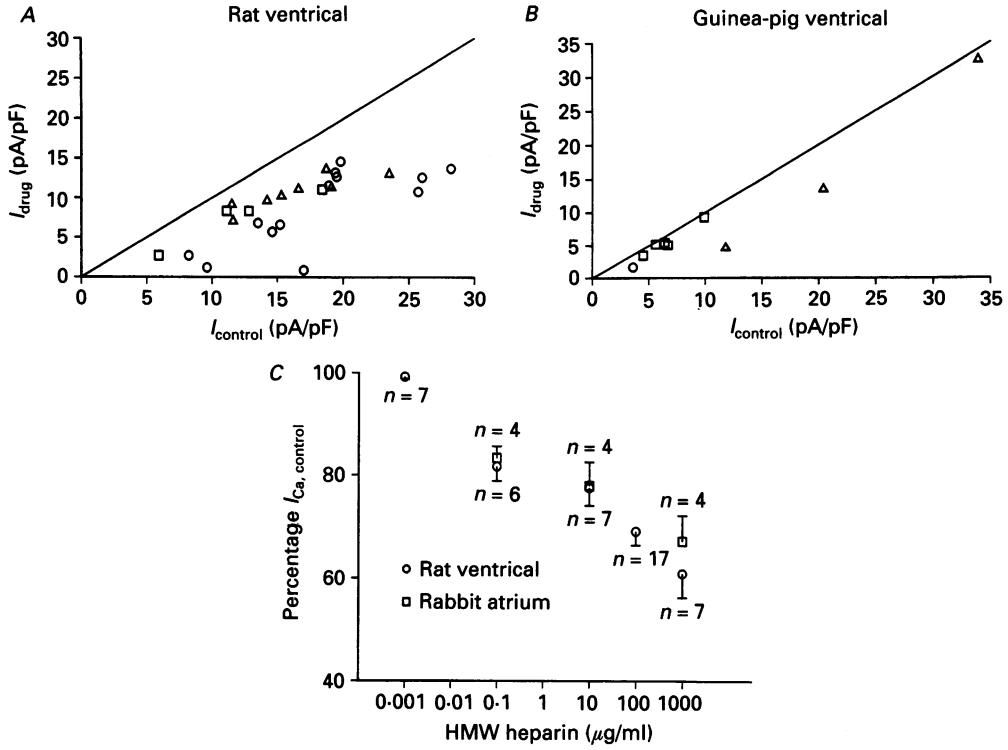

Fig. 3. The magnitude of the effect of heparin on I_{Ca}. The results obtained from all the rat (*A*) and guinea-pig (*B*) myocytes exposed to heparin in control solution (solution B) are compared. The amplitude of I_{Ca} in the presence of heparin is plotted *versus* the amplitude of the current under control conditions. Points below the unity line ($y = x$) represent suppression of I_{Ca} by heparin (○ represents clinical heparin, □ HMW and △ LMW heparin). *C*, dose dependence of HMW heparin-induced suppression of I_{Ca} in rat ventricular (○) and rabbit atrial (□) myocytes. Each point is labelled with a vertical bar representing the standard error of the mean determined from *n* cells.

No significant difference in the suppressive effects of LMW and HMW heparins on I_{Ca} was found. Clinical heparin in a 1:10 dilution seemed to be more efficient in suppression of I_{Ca}, but contains other ingredients such as sodium chloride and benzyl alcohol in variable concentrations and has a heparin activity specified only by its effect on coagulation. Thus clinical heparin was not used in evaluation of its mechanism of action. We used the HMW heparin in all the following experiments, since its biochemical characteristics on the Ca^{2+} channel have already been studied (Knaus *et al.* 1990).

Figure 3*C* shows the dose dependence of the heparin effect in cardiac myocytes. Generally, at a concentration of 0·001 μg/ml, HMW heparin failed to suppress I_{Ca}

but the effect was already highly significant at a concentration of 0·1 μg/ml HMW heparin corresponding to 6 nM or 0·017 USP unit/ml. Since 1 USP unit/ml is defined as the plasma concentration which effectively prevents coagulation this implies that some suppression of I_{Ca} is to be expected in the therapeutic range of the drug. The dose dependence of the suppressive effect of HMW heparin had a shallow slope and plateaued at 1 mg/ml ($\simeq$ 60 μM). Since the suppressive effect of heparin was only partially reversible, the measurements were done by using one concentration in each cell or by graded increases of the heparin concentrations without wash-out between two concentration steps. The results obtained by both procedures were similar (note small standard error bars). Similar results were obtained with cells from both rat ventricle and rabbit atrium (Fig. 3*C*). Measurements of dose-dependent effects of heparin were complicated by the slow run-down of Ca^{2+} current, and as such, provide only partial information on the heparin binding site.

Increasing $[Ca^{2+}]_o$ *does not suppress heparin effect*

Since heparin binds both monovalent and divalent cations (Mattai & Kwak, 1981, 1988), the apparent suppression of I_{Ca} could result secondary to a decrease in the effective activity of $[Ca^{2+}]_o$. To test for this possibility, we examined the effect of 100 μg/ml ($\simeq$ 6 μM) HMW heparin on rat ventricular myocyte in solutions containing 10 mM Ca^{2+}. Figure 4*A* and *B* shows that both the voltage dependence of I_{Ca} and the time course of its inactivation reflect the properties of high $[Ca^{2+}]_o$, i.e. I_{Ca} inactivates more rapidly and achieves its maximum at more positive potentials (cf. Fig. 2). Note, however, that neither the suppressive effect of heparin nor its voltage dependence were significantly altered in solutions with 10 mM Ca^{2+} ($n = 5$), suggesting that the binding of Ca^{2+} by heparin did not significantly reduce the extracellular Ca^{2+} activity. This finding is supported by spectrophotometric assays of $[Ca^{2+}]_o$ (see Methods). Figure 4*A* also shows that the suppression of I_{Ca} starts in less than 5 s, but is not complete before 70 s.

Comparison of heparin effect on the full time course of activation of I_{Ca} suggests that heparin may be more effective in suppressing the rapidly inactivating component of Ca^{2+} current.

The effect of heparin on I_{Ca} *persists during stimulation of G protein*

Because receptor-mediated pathways often involve regulatory G proteins, we tested to see if guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S; 50 μM) or guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S; 1 mM), when included in the internal pipette solution, alter the heparin-induced suppression of I_{Ca}. Figure 4*C* and *D* shows the time course of the suppressive effect of heparin on I_{Ca} using HMW heparin in a GTP-γ-S-dialysed cell. The suppression of I_{Ca} started immediately following superfusion of heparin and the time course of the effect and the magnitude of suppression were similar to those found under control conditions (cf. Fig. 2). I_{Ca} in GTP-γ-S-dialysed cells often recovered fully following the wash-out of heparin in contrast to the control cells, probably because of the slowing of the run-down phenomenon ($n = 8$). The presence of GDP-β-S in the pipette solution also did not change the heparin effect ($n = 4$). These findings suggest that G proteins are not likely to be involved in the suppression of I_{Ca} by heparin.

Modulation of the heparin effect by intracellular Ca^{2+} buffers

The results described so far were obtained in cells where the intracellular calcium concentration was strongly buffered by inclusion of 10 mM EGTA in the pipette solution. To approximate more physiological conditions we investigated the effect of

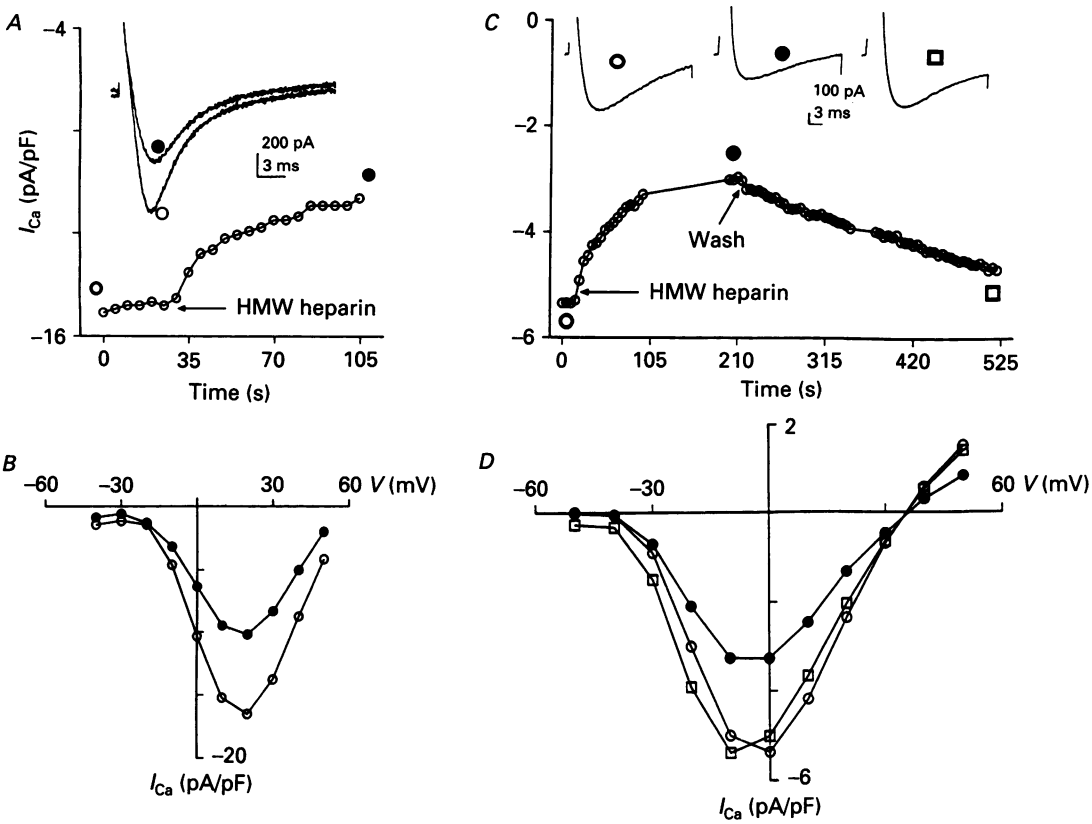

Fig. 4. The suppression of I_{Ca} by heparin persists in Ca^{2+}-rich solution (panels *A* and *B*) and in the presence of GTP-γ-S (panels *C* and *D*). Panel *A* shows the time course of the reduction of peak I_{Ca} after addition of 100 μg/ml HMW heparin (arrow) to a solution containing 10 mM Ca^{2+}. The current traces in the inset were recorded at the times indicated by ○ (control) and ● (heparin present). I_{Ca} was activated by depolarization to +20 mV. Panel *B* shows *I–V* relations measured just before (○) and after (●) the wash-in of heparin illustrated in panel *A* ($V_h = -50$ mV, $C_m = 68$ pF). Panel *C* shows the time course of heparin-induced changes in peak I_{Ca} measured while 50 μM GTP-γ-S was constantly present in the internal solution. I_{Ca} was activated by depolarization to 0 mV and is illustrated with sample traces shown as insets ($V_h = -90$ mV). Panel *D* shows *I–V* relations for I_{Ca} which, like the inset traces in panel *A*, were measured before (○), in the presence of (●) and after wash-out of (□) 100 μg/ml HMW heparin. In this experiment extracellular Na^+ was replaced by 137 mM Cs^+ to suppress sodium current (solution E in Table 1, $C_m = 122$ pF).

heparin when only 0·1 mM EGTA was included in the patch pipette solution. In such cells, which produced visible contractions and more rapidly inactivating Ca^{2+} currents, heparin continued to suppress I_{Ca}. In fact, Fig. 5 shows that the suppressive effect of heparin was more prominent on the rapidly inactivating component of I_{Ca}.

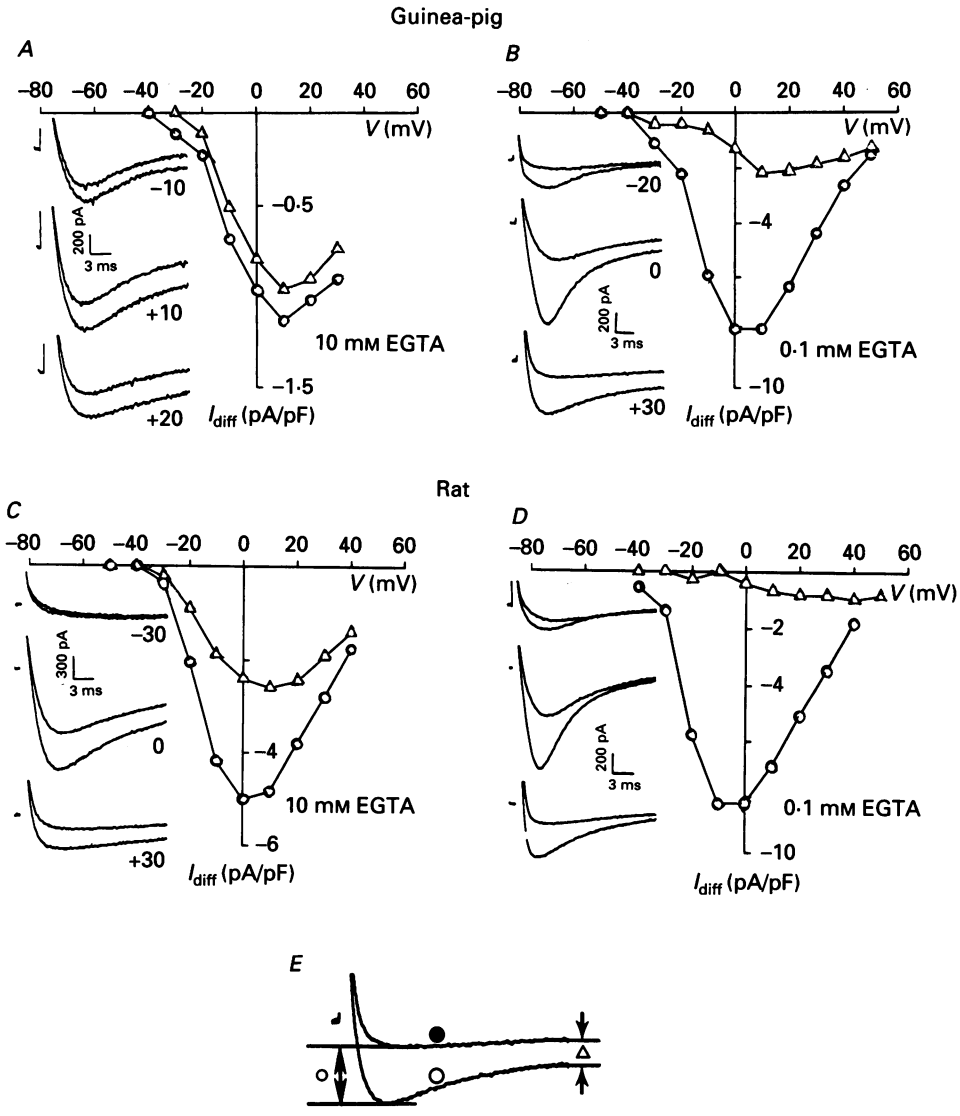

Fig. 5. Heparin suppresses the peak component of I_{Ca} more effectively than its maintained component. As shown in panel E, the effect of 100 μg/ml HMW heparin is quantified as the difference currents (○ − ●) measured at the peak of I_{Ca} (○) and at the end of the 30 ms voltage clamp depolarizations (△). Each of the panels *A* to *D* shows *I–V* relations for the heparin-induced changes in peak (○) and maintained (△) I_{Ca} as well as representative current records demonstrating the suppression of I_{Ca} at three different potentials (in mV). Panels *A* and *B* compare the heparin effect on the guinea-pig ventricular cells with 10 mM EGTA (panel *A*, $V_h = -60$ mV) and 0·1 mM EGTA (panel *B*, $V_h = -50$ mV) in the pipette solution. Panels *C* and *D* demonstrate the same type of experiments performed on rat ventricular myocytes with 10 mM EGTA (panel *C*, $V_h = -50$ mV) or 0·1 mM EGTA (panel *D*, $V_h = -60$ mV) in the pipette solution.

To illustrate the difference in the suppressive effects of heparin on the peak and the maintained currents in cells dialysed with 0·1 mM EGTA we plotted the voltage dependence of the heparin-induced difference currents. Comparison of the effects of heparin in cells with high (Fig. 5*A* and *C*) and low (Fig. 5*B* and *D*) concentrations

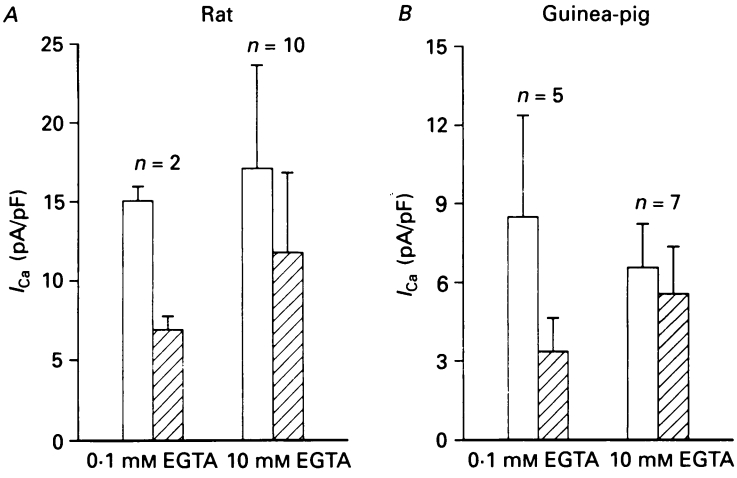

Fig. 6. Modulation of the heparin effect by buffering $[Ca^{2+}]_i$. Average values of peak I_{Ca} are compared in cells dialysed with 0·1 and 10 mM EGTA in rat (panel *A*) and guinea-pig (panel *B*) ventricular myocytes. Each experimental condition is represented by a pair of blocks which represents the peak value I_{Ca} averaged from n cells measured before (open) and after (hatched) exposure to heparin. Vertical bars indicate the standard deviations. cAMP (10 μM) was included in the dialysing solution. Notice that the quantity plotted in this figure is the peak value of I_{Ca} while Fig. 5 illustrates the same pool of experiments but emphasizes the heparin-induced difference currents.

of EGTA shows a remarkable difference in the degree of suppression of I_{Ca}. Notice that the maintained component of I_{Ca} (△) is reduced much less than the peak value of I_{Ca} (○) when the EGTA concentration is 0·1 mM (Fig. 5*B* and *D*). In cells with 10 mM EGTA, on the other hand, there is little difference between the suppression of peak and maintained current (Fig. 5*A* and *C*). Some species differences were noted. Thus, it was found that the maintained component of I_{Ca} was more insensitive to heparin in rat (Fig. 5*C* and *D*) than in guinea-pig (Fig. 5*A* and *B*) ventricular myocytes. This species dependence could be linked to the extent to which effective calcium buffering is achieved in the two cell types. Consistent with this idea, EGTA was more effective in suppressing the rate of inactivation of the Ca^{2+} channel in guinea-pig than in rat myocytes. The degree of intracellular Ca^{2+} buffering, on the other hand, did not change the time course of the suppressive effect of heparin which started within seconds, developed within one or two minutes and reversed very slowly upon wash-out (not shown).

We compared the amplitude of the average peak I_{Ca} before and after exposure to HMW heparin in rat and guinea-pig ventricular myocytes buffered with high (10 mM) and low (0·1 mM) concentrations of EGTA (Fig. 6). Results from both rat (panel *A*) and guinea-pig (panel *B*) myocytes show that the heparin-induced

suppression of peak I_{Ca} was significantly larger, about 60%, in cells dialysed with 0·1 mM EGTA than the value of about 30% or less, seen in cells with 10 mM EGTA. The current traces in Fig. 5 are representative of these average values. The averaged currents also suggest that heparin, in high EGTA-buffered (10 mM) cells, may be more effective in suppressing I_{Ca} in rat than in guinea-pig ventricular myocytes (see also Fig. 3*A* and *B*). This was found to be highly significant since the average reduction of I_{Ca} was $32{\cdot}5 \pm 3{\cdot}7$% (S.E.M., $n = 10$) in rat myocytes but only $15{\cdot}2 \pm 2{\cdot}9$% (S.E.M., $n = 7$) in guinea-pig myocytes.

Effect of heparin on Ba^{2+} currents through the Ca^{2+} channels

The above results might suggest that the suppressive effect of heparin may be mediated by a molecular mechanism related to the calcium-induced inactivation process of the Ca^{2+} channel. To test this possibility Ca^{2+} was replaced by Ba^{2+} as a charge carrier. EGTA (100 μM) was added to the Ba^{2+}-containing Tyrode solution to bind possible contaminant Ca^{2+} concentrations (Katzka & Morad, 1991). Figure 7 shows that although the time course of inactivation of the Ca^{2+} channel was significantly slowed when Ba^{2+} was the charge carrier, the current through the channel was still suppressed by about 35% in a manner quite similar to that observed in high EGTA-buffered cells (e.g. Fig. 1). Neither the time course of the heparin effect (Fig. 7*A*), nor the kinetics of I_{Ba} were significantly altered (insets in Fig. 7*B*). The suppressive effect of heparin was neither voltage dependent (Fig. 7*B*), nor did it alter the gating parameters of the channel (Fig. 7*C*, $n = 6$).

These results are quantitatively similar to the results obtained for I_{Ca} with 10 mM EGTA inside the cell. The data suggest that although the suppressive effect of heparin is more specific to Ca^{2+} it is not exclusively mediated by the Ca^{2+}-induced inactivation process.

Possible site of action of heparin

Since heparin appears to bind directly to the L-type Ca^{2+} channel (Knaus *et al.* 1990), displacing binding of dihydropyridines, benzothiazepines and phenylalkylamines, we examined whether the suppressive effect of heparin could be altered in the presence of other well-known modifiers of the Ca^{2+} channel. The ability of heparin to antagonize the isoprenaline-induced enhancement of adenylate cyclase activity (Cutler & Christian, 1984) was tested by examining the effect of heparin following stimulation of β-receptor or vice versa. Isoprenaline continued to enhance I_{Ca} even though heparin had suppressed I_{Ca} (not shown). Similarly, it was found that heparin still reduced I_{Ca} after its enhancement by isoprenaline. Thus we found no indication of interaction between the β-receptor and the heparin binding site.

Phorbol esters recently have been shown to modulate neuronal (Hockberger, Toselli, Swandula & Lux, 1989) and cardiac (L. Lacinova & M. Morad, unpublished observations) calcium current. Phorbol-12,13-diacetate (PDA; 10 nM) and its analogues applied extracellularly appear to suppress calcium current rapidly ($t_{\frac{1}{2}} \simeq 200$ ms, Hockberger *et al.* 1989), consistent with the suggested extracellular site of action. We tested, therefore, if heparin and PDA might display the type of drug interaction which might indicate a common site of action. Although PDA applied extracellularly rapidly suppressed I_{Ca}, it did not alter the suppressive effect

of heparin on I_{Ca} (not shown). Thus, the suppressive effects of heparin and PDA were additive and independent of each other, suggesting that they acted at different membrane sites.

Extracellular ionic content vs. heparin effect

Since the results suggest that the suppressive effect of heparin on I_{Ca} may be mediated by an extracellular binding site somehow related to the gating of the

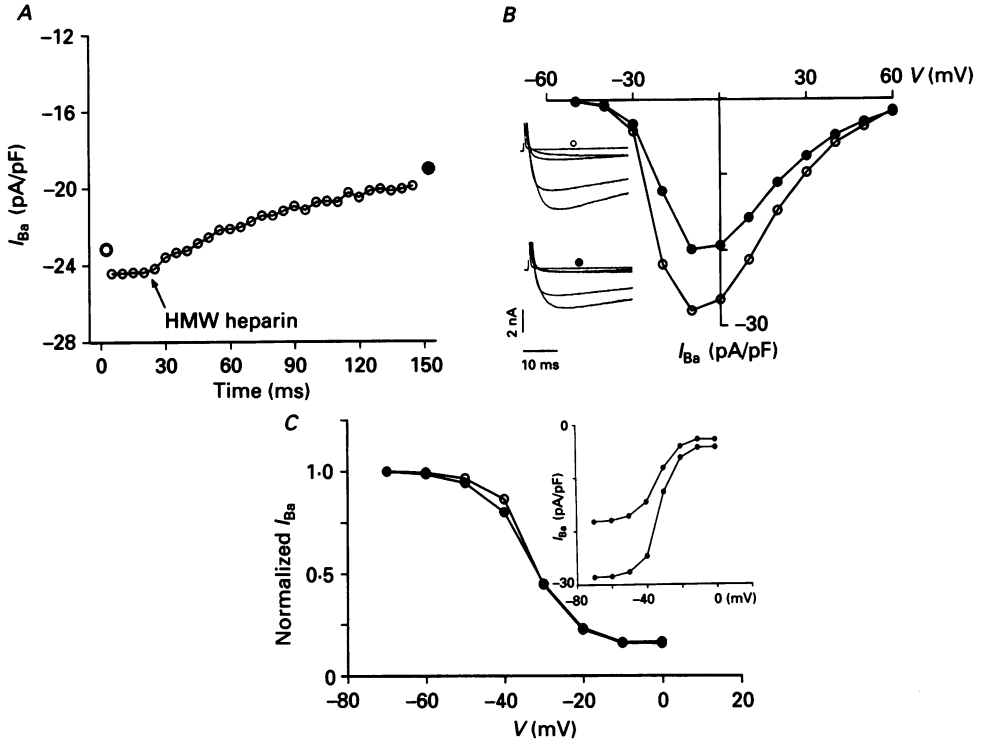

Fig. 7. Heparin suppresses Ba^{2+} current through the Ca^{2+} channel. Panel *A* shows the time course of the heparin effect on the peak inward current (I_{Ba}) recorded in a rat ventricular myocyte with 2 mM Ba^{2+}, no Ca^{2+}, and 0·1 mM EGTA in the external solution. The effect of heparin was analysed by measuring the current–voltage relation (*B*) and steady-state availability curve (*C*) before (○) and after (●) addition of 100 μg/ml HMW heparin. Panel *B* shows relations and, as insets, original current records obtained by depolarization to −50, −30, −10, 10 and 30 mV (V_h = −60 mV). Panel *C* shows the availability curves for I_{Ca} scaled to unity at −70 mV, as well as insets with the unscaled currents (C_m = 198 pF).

rapidly inactivating component of the Ca^{2+} channel, we examined the possible role of extracellular Na^+ and Mg^{2+}, known to alter channel gating, in mediating the heparin effect. We tested the heparin effect in six different external solutions (Table 1). Removal of only Na^+ (solution E) or only Mg^{2+} (solution A) from the control solution (solution B) had little, if any, effect on the time course or the degree to which heparin suppressed I_{Ca} (not shown). However, when both Na^+ and Mg^{2+} were removed (solution D), the blocking effect of heparin was strongly potentiated.

Figure 8*A* shows that the onset of the suppressive effect of heparin was markedly enhanced and was almost complete within 10 s. Reversal of the heparin effect was also very rapid and more complete. Under these conditions the voltage dependence of peak I_{Ca} and the steady-state inactivation were shifted by about 10 mV toward more negative potentials (Fig. 8*B* and *C*).

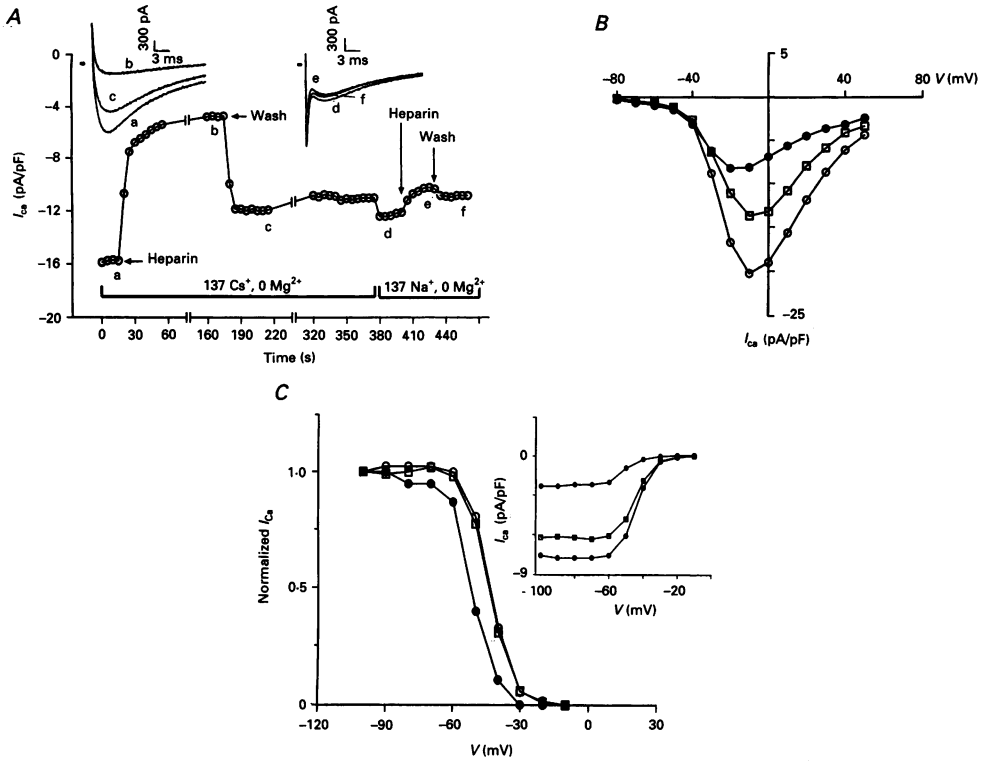

Fig. 8. Removal of extracellular Na^+ and Mg^{2+} potentiates the suppressive effect of heparin. Panel *A* shows the time course of changes in peak I_{Ca} activated by depolarizing pulses to 0 mV in Na^+- and/or Mg^{2+}-free solutions. The additions of 100 μg/ml HMW heparin to a solution devoid of both Na^+ and Mg^{2+} markedly and reversibly suppressed I_{Ca} (0 to 220 s, arrows; solution D, Table 1). Similar concentrations of heparin had no significant suppressive effect when Na^+, but not Mg^{2+}, was readmitted (380 to 470 s, arrows; solution A, Table 1). Original tracings of I_{Ca} measured in the times indicated by letters a–f are shown in the insets. The effect of heparin in Na^+- and Mg^{2+}-free solution (solution D) was analysed by measuring the *I–V* relation (*B*) and the availability curve (*C*) under control conditions (○), in presence 100 μg/ml HMW heparin (●) and after washout (□). The availability curves, scaled to unity at −100 mV, show that heparin shifts the steady-state inactivation toward more negative potentials in the absence of Mg^{2+} and Na^+. The unscaled availability curves are shown in the inset. ($V_h = -90$ mV, $C_m = 160$ pF).

Solutions C and F, containing 10 mM Mg^{2+}, suppressed I_{Ca} by themselves and shifted the steady-state inactivation curve by about 20 mV toward more positive potentials (Kass & Krafte, 1987; White & Hartzell, 1989). In the absence of Na^+, where 10 mM Mg^{2+} suppressed I_{Ca} to 32·0 ± 2·7 % (S.E.M., $n = 6$) of its initial value, it

was found that heparin increased I_{Ca} (Fig. 9). This heparin-induced increase only partially relieved the Mg^{2+}-induced suppression as I_{Ca} only recovered to 48·6 ± 3·3 % (S.E.M., $n = 6$) of its initial value. Similarly, heparin shifted the steady-state inactivation parameters by about 10 mV toward more negative potentials thereby

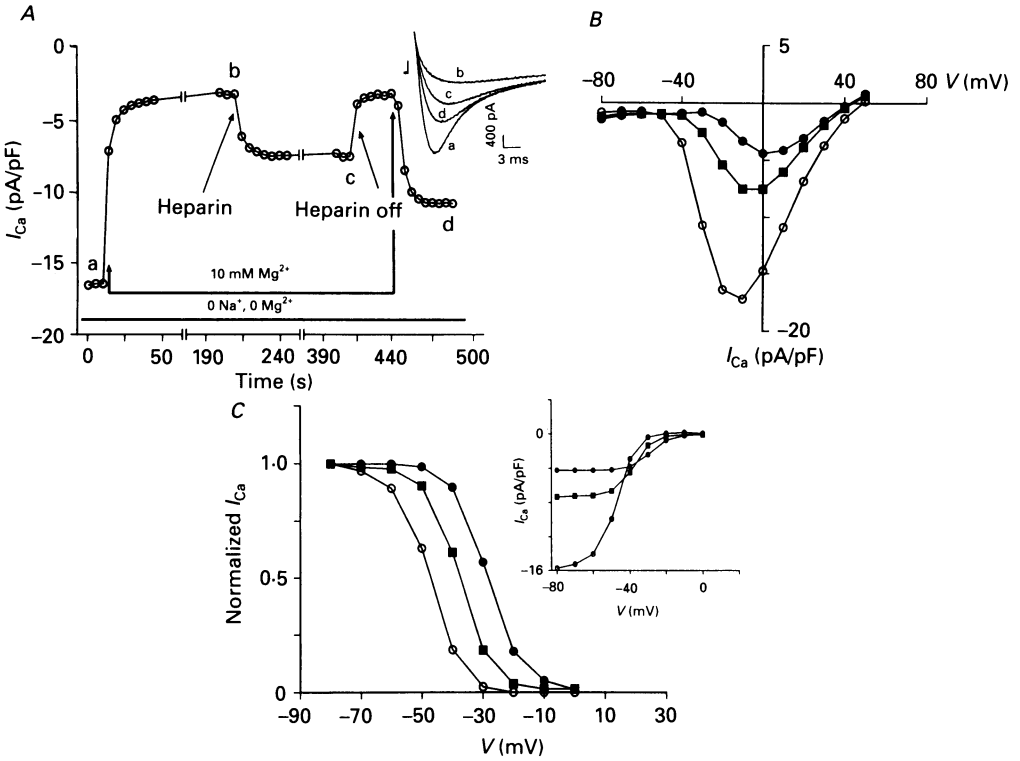

Fig. 9. Heparin partially relieves the block of I_{Ca} caused by 10 mM $[Mg^{2+}]_o$. Panel *A*, in Na^+-free solution I_{Ca}, measured at −10 mV, is suppressed by addition of 10 mM Mg^{2+} (arrow, solution F, Table 1) but partially recovers on addition of 100 μg/ml HMW heparin (Heparin). Sample recordings of I_{Ca}, measured at the indicated times, are labelled by letters a–d. Panel *B* shows the voltage dependence of I_{Ca} in Na^+- and Mg^{2+}-free solution (○), after addition of 10 mM Mg^{2+} (●) and after further addition of 100 μg/ml HMW heparin in the presence of 10 mM Mg^{2+} (■). Panel *C* shows steady-state availability curves measured in the same cell with test pulses to 0 mV. The availability curves were normalized to unity at −80 mV and are shown in the inset without normalization. The Mg^{2+}-induced shift of the normalized steady-state inactivation curve, toward more positive potentials, was partially reversed by heparin. ($V_h = -90$ mV, $C_m = 182$ pF).

partially reversing the Mg^{2+}-induced shift (Fig. 9). In Na^+-based Tyrode solution containing 10 mM Mg^{2+} (solution C), however, heparin failed to alter the Ca^{2+} current significantly.

Heparin affected not only the amplitude of I_{Ca}, but also the shape of the current–voltage relations and the steady-state inactivation parameters. The extent of all these effects depends on cationic composition of extracellular solution and are reviewed in Table 1. Heparin shifted the steady-state inactivation curve toward more negative potentials only when Na^+ was removed from extracellular solution

(see Table 1, also compare Figs 2, 7, 8 and 9). This finding suggests electrostatic interaction with membrane surface charges which may be screened differently by Na^+ and Cs^+.

Specificity of heparin effect on I_{Ca}

We also tested the effect of 100 μg/ml dextran sulphate, another polysaccharide, which has similar chemical structure and charge and shows an effect on cell aggregation similar to that of heparin (Thurn & Underhill, 1986). Further, dextran sulphate is more effective than heparin in inhibiting the adenylate cyclase activity of isolated membranes of rat salivary gland (Cutler & Christian, 1984). Both of these dextran effects are probably related to the ionic interaction with the surface charge of cell membranes. In the control bathing solutions (solution B) dextran sulphate suppressed I_{Ca} by only 10% (not shown), and the effect was irreversible. In solution D (0 mM Na^+, 0 mM Mg^{2+}) where the suppressive effect of heparin was large, rapid and reversible (see Fig. 8), the effect of dextran sulphate was again ineffective as it suppressed I_{Ca} by no more than 10%. The contrast between these two polysaccharides was even more remarkable in solution F (0 mM Na^+, 10 mM Mg^{2+}), where dextran sulphate failed to have any effect on I_{Ca} (not shown). These results suggest that the blocking of the Ca^{2+} channel by heparin is not a common feature of sulphated polysaccharides.

Since HMW heparin at a concentration of 100 μg/ml contains about 0·5 mM Na^+ (Methods), we tested to find if the reversal of Mg^{2+}-induced block of I_{Ca} could be caused by this Na^+ concentration. Neither 0·5 nor 5 mM Na^+ by itself exerted any change in the amplitude, I–V relation or steady-state inactivation curve of I_{Ca} (not shown). When 5 mM Na^+ was added to a Na^+-free solution with 10 mM Mg^{2+} (solution F), a small I_{Na} was activated, but I_{Ca} did not change significantly.

These data suggest that the above described effects of heparin are specifically related to HMW heparin and not to contaminant Na^+ concentrations, and that these properties are not shared by other polysaccharides.

DISCUSSION

The major finding of this report is that heparin suppresses Ca^{2+} channels. This effect is highly reproducible, exhibits some species dependence, and can be potentiated or reduced depending in part on the ionic components of the solutions. Although the mechanism of action remains to be worked out we have considered a variety of possibilities ranging from changes in ionic concentrations (volume effects), and modulation of surface potentials (surface effect), to binding to sites controlling regulatory pathways or located directly on the Ca^{2+} channel protein.

Does heparin bind cations in sufficient quantity to significantly alter the ionic concentrations?

Heparin binds large amounts of both monovalent and divalent cations (Mattai & Kwak, 1981, 1988). It may be suggested, therefore that the suppression of I_{Ca} might be mediated by a reduction of $[Ca^{2+}]_o$ produced by binding of several Ca^{2+} ions to each heparin molecule. Similarly, the partial reversal of the Mg^{2+}-induced suppression

of I_{Ca} (Fig. 9) might reflect the ability of heparin to chelate Mg^{2+}. There are several arguments against such an interpretation: (1) using tetramethyl murexide as a Ca^{2+}-sensing dye we found that 100 μg/ml HMW heparin at most would reduce $[Ca^{2+}]_o$ by 4% (from 2 to 1·92 mM, Fig. 1*A*) while I_{Ca} was reduced, typically, by 30% (Table 1); (2) heparin continued to suppress I_{Ca} even when the extracellular solution contained 10 mM Ca^{2+} (Fig. 4) and/or when Ba^{2+} (which has a lower binding affinity for heparin; Mattai & Kwak, 1988) was the carrier of charge through the Ca^{2+} channel (Fig. 7); (3) heparin has a lower affinity for Mg^{2+} than for Ca^{2+} (Fig. 1); (4) the suppression of I_{Ca} by heparin typically took effect over several tenths of seconds and was normally only partially reversible (Figs 2, 4 and 7) while the heparin-containing solutions were added or removed within a fraction of a second (and were pre-equilibrated often for hours prior to their use); and (5) the effect of heparin was noticeably different when Na^+ in the extracellular solution was replaced by Cs^+, yet such a change had no detectable effect on the binding of Ca^{2+} and Mg^{2+} to heparin (Table 1). We conclude, therefore, that changes in $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ do not pose a serious problem when moderate concentrations of heparin are tested. Such problems are likely to occur only when heparin is used as the Ca^{2+} salt in large concentrations ($\gg$ 100 μg/ml). The somewhat puzzling lack of an effect of Ca-heparin on I_{Ca} should be tested, therefore, only under conditions where $[Ca^{2+}]_o$ is carefully buffered or adjusted, e.g. by spectrophotometric titration.

Does heparin alter the surface potential of the cells?

Polyvalent ions are known to alter the electric field in the immediate vicinity of the membrane by a mechanism which may be non-specific, depending only on valence and concentration, or may involve binding sites with well defined selectivities. In either case the characteristic effect is a uniform shift of activation and inactivation parameters along the voltage axis. Considering the large negative charge of heparin, it may be questioned whether the effects of heparin are mediated in part by alterations in membrane surface charge effects. There is some evidence for this hypothesis as heparin, in Na^+-free solutions, shifted both the steady-state availability curve of I_{Ca} and the peak of its voltage dependence about 10 mV toward more negative potentials (Figs 8 and 9, Table 1). These shifts are in the opposite direction to those of 10 mM Ca^{2+} (cf. Figs 2 and 4) or Mg^{2+} (Fig. 9). The major effects of heparin, however, cannot be explained by alterations of the membrane surface charge. The suppression of I_{Ca} in Na^+-based solutions occurred, for instance, without detectable shifts in the voltage dependence or availability of the channel (Figs 2, 4 and 7, Table 1). Furthermore, we found that dextran, in spite of its similarities in structure and charge, did not mimic the effect of heparin on I_{Ca}. These findings do not support the non-specific effects of membrane surface charge in mediating the heparin response.

Where does the heparin molecule bind?

Since the β-adrenergic enhancement of I_{Ca} remained unaltered in heparin-treated myocytes it was unlikely that heparin interferes strongly with the pathway leading to protein kinase A-mediated phosphorylation of the Ca^{2+} channel. Similarly the persistence of the heparin effect in the presence of phorbol esters, an activator of

protein kinase C, suggests also no involvement of this phosphorylation pathway. The relatively slow suppression of I_{Ca} by heparin might suggest possible receptor-mediated activation of the intracellular second messenger system via the regulatory G proteins. The involvement of G proteins was unlikely, however, as addition of GTP-γ-S or GDP-β-S to the dialysing solutions did not alter heparin-induced suppression of I_{Ca} (Fig. 4*C* and *D*). In short, we found no compelling evidence that the Ca^{2+} channel suppressive effects of heparin were mediated by some of the common regulatory pathways involved in the control of cardiac excitability.

The modification of the heparin effect with variations in external cation content (Na^+, Cs^+ and Mg^{2+}) was, on the other hand, consistent with the possible binding of heparin to a molecular moiety near the mouth of the Ca^{2+} channel where cationic sites are thought to be located. Another characteristic effect of heparin was its strong suppression of the rapidly inactivating component of I_{Ca}. This current component, and its suppression by heparin, was seen more clearly in cells where lowered concentrations of EGTA allowed Ca^{2+}-induced inactivation of I_{Ca} to take its course (Figs 5 and 6). This finding may suggest that heparin, which does not easily permeate across the membrane, exerts its effect allosterically on the Ca^{2+}-induced inactivation process.

A direct effect of heparin on the Ca^{2+} channel is consistent with the finding that heparin binds to the isolated L-type Ca^{2+} channel displacing phenylalkylamine, benzothiazepine and 1,4-dihydropyridine (Knaus *et al.* 1990). The dose dependence of the heparin effect shows that the apparent affinity of heparin to the Ca^{2+} channel in native membrane (Fig. 3*C*) is lower than that of the purified channel. This may possibly result from limited diffusivity of the highly charged heparin molecule in the T-tubules of the myocytes. The major disagreement with the previous study was, however, that heparin suppressed instead of enhancing the I_{Ca} independent of the type of heparin and the species of animals used (Fig. 3). The reason for this discrepancy is not readily apparent, as we attempted to reproduce the exact experimental conditions, including the source and the supplier of heparin, the type of myocytes and the intracellular and extracellular solutions used. The only conditions where we found an increase in I_{Ca} was (1) when the Ca^{2+}-salt of heparin was added in sufficient quantity to significantly increase $[Ca^{2+}]_o$ and (2) when heparin partially relieved the Mg^{2+}-induced block in Cs^+-based Tyrode solution (Fig. 9). Neither of these conditions would appear to be of immediate relevance to the resolution of the discrepancy. It is of interest though that the heparin effect can be significantly altered simply by changing the ionic concentrations. This may indicate that a clearer understanding of ionic permeation in general, and of the heparin effect in particular, may be obtained by studying the detailed distribution of electric charge at or near the opening of the Ca^{2+} channel.

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