Mechanisms of the Contractile Effects of Levosimendan in the Mammalian Heart

PETER BOKNIK, JOACHIM NEUMANN, GRIT KASPAREIT, WILHELM SCHMITZ, HASSO SCHOLZ, UTE VAHLENSIECK and NORBERT ZIMMERMANN

Institut für Pharmakologie und Toxikologie der Westfälischen Wilhelms-Universität, Münster, Federal Republic of Germany

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ABSTRACT

In spontaneously beating guinea pig right atria, levosimendan (LS, or R-[[1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl]-phenyl]-hydrazono]propanedinitrile) exerted a positive chronotropic effect starting at 0.1 μM. In electrically driven guinea pig left atria, LS (0.1–10 μM) increased force of contraction without changing time parameters of contraction. In electrically driven right papillary muscles, LS (0.1–10 μM) enhanced force of contraction without affecting time parameters of contraction. The maximal effect on force of contraction at 10 μM amounted to 130 ± 8.6% of predrug value. The positive inotropic effect of LS in papillary muscles was greatly diminished by additionally applied carbachol. In [32P]-labeled guinea pig ventricular cardiomyocytes, LS increased the phosphorylation state of phospholamban, the inhibitory subunit of troponin and C-protein. The maximal effect at 1 μM amounted to 134 ± 8.6%, 124 ± 4.2% and 121 ± 8% of control for phospholamban, the inhibitory subunit of troponin and C-protein, respectively. LS (1 μM) increased cAMP content from 6.3 ± 0.3 to 8.1 ± 0.3 pmol/mg protein in guinea pig ventricular cardiomyocytes. Furthermore, whole-cell patch-clamp studies were performed in guinea pig ventricular cardiomyocytes. In this setup, 10 μM LS increased the amplitude of L-type Ca2+ current to 402 ± 86% of predrug value.

The positive inotropic effects of cAMP-increasing agents, like beta adrenoreceptor agonists and PDE inhibitors, are greatly diminished in isolated preparations from failing human hearts (Bristow et al., 1982; Feldman et al., 1987; Steinfath et al., 1992; Schmitz et al., 1992). Thus other positive inotropic principles have been sought. One intuitively appealing mechanism is to sensitize the myofilaments to calcium. Such compounds, calcium sensitizers, shift the calcium-force relationship in isolated myofilaments to the left (toward lower concentrations of calcium; for review see Lee and Allen, 1995). Hence calcium sensitizers should be able to increase cardiac force of contraction independently of the adenyl cyclase pathway. One of the first calcium sensitizers discovered was pimobendan. However, pimobendan proved to be a much more potent PDE inhibitor than a calcium sensitizer (Fujino et al., 1988). Next to be developed was EMD 57033, which appeared to be more promising because it was similarly potent as a calcium sensitizer and as a PDE inhibitor (Lues et al., 1993). The PDE inhibitory effect of EMD 57033 was relevant because it increased cAMP levels in cardiomyocytes (Lues et al., 1993) and led to the phosphorylation of target proteins for the cAMP-dependent protein kinase (namely PLB and Tnl; Neumann et al., 1995c).

CGP 48506 may represent a major step forward (Herold et al., 1995). This is the first calcium sensitizer discovered to be devoid of phosphodiesterase inhibitory activity in the guinea pig heart (Zimmermann et al., 1996a) and in the human heart (Neumann et al., 1996). Accordingly, it did not increase the cAMP content in guinea pig ventricular cardiomyocytes (Zimmermann et al., 1996a).

The exact mechanism of action of CGP 48506 is unknown at present. It seems to act directly on the thin or thick filaments. However, it does not act via binding to troponin C (Wolska et al., 1996). However, a compound that binds to and probably acts via troponin C is available: LS (Pollesello et al., 1994). LS is actually the only calcium sensitizer with a well-understood mechanism of action. We owe this understanding to the screening assay. The screening system was an affinity column to which troponin C was coupled (Pollesello et al., 1994). Only drugs that were bound firmly to troponin C remained on the affinity column. In this system, racemic

ABBREVIATIONS: CP, C-protein; DMSO, dimethyl sulfoxide; LS, levosimendan; MLC, myosin light chains; PDE, phosphodiesterase; PLB, phospholamban; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tnl, inhibitory subunit of troponin; GPVC, [32P]-labeled guinea pig ventricular cardiomyocytes.
simendan was discovered (Haikala et al., 1995). The active enantiomer was purified and called LS, and it was used in subsequent experiments. It exerted positive inotropic effects in isolated cardiac preparations such as Langendorff hearts (Edes et al., 1995). Despite the selective binding to troponin C, it was quickly discovered that LS is also a potent PDE inhibitor. It inhibits cardiac PDE isoenzymes, among them PDE type III (Raasmaja et al., 1991), which is thought to be functionally linked to inotropy (Brunnhorst et al., 1989; Bethke et al., 1991; 1992).

However, it is not known whether PDE inhibitory effects contribute to the positive inotropic effect of LS in relevant manner. Therefore, we studied to what extent the PDE inhibitory action of LS is functionally relevant. That is, does it act mainly as a calcium sensitizer like CGP 48506 or mainly by increasing cellular cAMP content like pimobendan? To that end, in an integrative approach we studied the effects of LS on rate and force of contraction, on cAMP content, on the L-type Ca ++ current and on the phosphorylation of cardiac regulatory proteins such as PLB. Here we present evidence that a cAMP increase distinctly contributes to the cardiac effects of LS and therefore its value for the treatment of heart failure is questionable.

Materials and Methods

Contraction experiments. Contraction experiments were performed as described previously (Böhm et al., 1984). In brief, right atria, left atria and papillary muscles from right ventricles were isolated from hearts of reserpinized (5 mg/kg, 16 h before sacrifice) or nonreserpinized male guinea pigs (300–400 g). The bathing solution consisted of (in mM) NaCl 119.8, KCl 5.4, CaCl 2, 1.8, MgCl 2 1.5, NaH 2 PO 4 0.42, NaHCO 3 22.6, ethylenedinitrilotetraacetic acid disodium salt (Na 2 EDTA) 0.05, ascorbic acid 0.28 and glucose 5.0, continuously gassed with 95% O 2 and 5% CO 2 and maintained at 35°C, resulting in a pH of 7.4. Isometric force of contraction was measured after preloading each muscle to optimal length. Papillary muscles and left atria were electrically stimulated at 1 Hz with rectangular pulses 5 ms in duration (Grass stimulus SD9; Grass, Quincy, MA); the voltage was about 10% to 20% greater than the threshold. Preparations were allowed to contract until equilibrium was reached (at least 30 min) before 1 µg/ml adenosine deaminase was added for an additional 30 min. Adenosine deaminase was employed to degrade endogenous adenosine, which can interfere with the effect of cAMP-increasing agents (Heller et al., 1989; Gupta et al., 1993). LS or the solvent DMSO was added cumulatively (allowing 30 min for each concentration). Isoproterenol was dissolved in Tyrode’s solution and cumulatively applied (allowing 5 min for each concentration). CGP 48506 was also applied cumulatively (allowing 30 min for each concentration). In additional experiments, preparations were first stimulated with LS (0.1 µM) for 30 min, and thereafter (after steady state was reached) isoproterenol was cumulatively added. The time course of contraction was evaluated using twitches recorded at high chart speed.

Isolation of cardiomyocytes for phosphorylation experiments. Guinea pig ventricular cardiomyocytes were isolated as described (Neumann et al., 1993).

Labeling of cardiomyocytes and protein phosphorylation. Three milliliters of a gravity-settled suspension of freshly isolated cardiomyocytes was incubated at 37°C with 5 mCi of [32P]-labeled orthophosphate in 5 ml of solution A consisting of (in mM) NaCl 132.0, KCl 4.8, CaCl 2 1.8, MgSO 4 1.2, glucose 10.0, HEPES 10.0 and sodium pyruvate 2.5; pH was adjusted to 7.4. After 60 min, cardiomyocytes were washed with 10 ml of solution A by allowing cells to settle by gravity. Finally, gravity-settled cardiomyocytes were diluted 5-fold in solution A, and the resulting cell suspension was used in phosphorylation experiments. For phosphorylation experiments, adenosine deaminase (10 U/ml) was added to avoid interference from endogenous adenosine upon treatment (Gupta et al., 1993). The drug solution (150 µl) was preincubated at 37°C for 2 min before mixing with 150 µl of the diluted cardiomyocytes, kept at 37°C. After 30 min, reaction was stopped by adding 150 µl SDS stop solution (Laemmli, 1970), which consisted of Tris(hydroxymethyl)aminomethane 62.5 mM, SDS 10% (w/v), glycerol 10% (w/v), DL-dithiothreitol 0.6% (w/v) and a trace of bromophenol blue; pH was adjusted to 6.8. Samples were frozen at −20°C. SDS-PAGE was performed using 10% polyacrylamide separating gels with 4% stacking gels or using 5% separating gels with 3% stacking gels according to Neumann et al. (1995a): In brief, samples were thawed and then heat-treated for 10 min at 95°C in order to convert the high-molecular-weight form of PLB into the low-molecular-weight form. An aliquot of 100 µl, corresponding to 70 to 80 µg protein, was applied on each lane. Gels were run, dried and subjected to autoradiography. Gel pieces identified by overlay of autoradiograms as containing the proteins of interest were excised, and radioactivity was quantified in a liquid scintillation counter as described (e.g., Neumann et al., 1993).

Determination of cAMP. The cAMP content was measured by means of radioimmunoassay as described previously (Neumann et al., 1993). Protein was determined according to Bradford (1976).

Electrophysiologicalexperiments. Electrophysiologicalexperiments were done as described (Herzig et al., 1995). Guinea pig ventricular myocytes were isolated by a collagenase/protease digestion of Langendorff-perfused hearts (37°C, 52 mm Hg) from male guinea pigs (250–350 g). After 5 min of perfusion with calcium-free Tyrode’s solution (in mM: NaCl 135.0, KCl 4.0, NaH 2 PO 4 0.3, MgCl 2 1.0, HEPES 10.0 and dextrose 10.0, pH 7.4), enzymes were added to this solution at a flow-independent dose rate of 1.4 mg/min collagenase (type 1, Worthington, Freehold, NJ) and 0.6 mg/min protease (type XIV, SIGMA, Deisenhofen, Germany) over a period of 5 min, using an infusion pump. Afterwards the hearts were perfused for 10 min with enzyme-free Tyrode’s solution containing 0.2 mM calcium. Cells were harvested after mincing of the hearts with fine scissors, gentle agitation of the tissue and filtering through a nylon mesh. Cells were plated in petri dishes, which served as recording chambers (volume approximately 1 ml) on the stage of an inverted microscope (Leica, Köln, Germany). Whole-cell patch-clamp recordings were performed in “physiological” solutions in order to avoid any confounding effects of artificial conditions on intracellular signal transduction. Tyrode’s solution (see above, but containing 2 mM CaCl 2, 22°C–24°C) served as the extracellular solution, and recording pipettes (soft glass coated with Sylgard, 1.2–2.5 MΩ) were filled with (in mM: K + aspartate 80.0, KCl 5.0, KH 2 PO 4 10.0, MgCl 2 0.5, MgATP 3.0, HEPES 5.0, ethylene glycol bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid (EGTA) 1.0, pH 7.4. L-type calcium currents were elicited by voltage steps from a holding potential of −40 mV to a test potential of +10 mV for 300 ms, applied every 10 s. Current was recorded using an L/M-PC amplifier (LIST-Electronic, Darmstadt, Germany) connected to a 486 computer that was equipped with the ISO2 software (version 1.2, MFK, Niedernhausen, Germany). Currents were evaluated as the difference between peak inward current and the current level at the end of the test pulse. Series resistance was compensated to the maximal possible extent, using the feedback circuitry of the amplifier.

Protocols in electrophysiologicalexperiments. LS was dissolved in DMSO (10 mM), isoproterenol in twice-distilled water (10 mM) containing 1 mg/ml ascorbic acid to prevent oxidation. After an equilibration period of at least 5 min, cells were superfused with Tyrode’s solution containing 10 µM LS or 0.01 µM isoproterenol at a rate of 60 ml/h. After stabilization of maximal effect, cells were superfused with drug-free Tyrode’s solution (washout).

Chemicals. Substances used were adenosine deaminase, collagenase A (Boehringer Mannheim, Germany), bovine serum albumin, protease type XIV (Sigma, Deisenhofen, Germany), collagenase type
Results

Contractile studies in right atria. In isolated, spontaneously beating right atria from reserpinized guinea pigs, the effects of increasing concentrations of LS cumulatively applied were measured. LS at 0.1 μM, 1 μM and 10 μM, but not at 100 μM, increased frequency (fig. 1). Similar effects were found in atria from nonreserpinized guinea pigs (data not shown). Isoproterenol increased frequency by 85% (5.3 Hz) at 1 μM (n = 4). For comparison, the “pure” calcium sensitizer CGP 48506 was studied; 10 μM and 30 μM did not affect frequency, and 100 μM CGP 48506 slightly (by 11%) reduced frequency, from 2.7 ± 0.1 Hz to 2.4 ± 0.2 Hz (n = 4, P < .05).

Contractile studies in left atria. In isolated, electrically driven left atria from reserpinized guinea pigs, the effect of increasing concentrations of LS applied cumulatively were measured. LS increased force of contraction at 0.1 μM, 1 μM and 10 μM, and 10 μM increased force of contraction to 250% of the predrug value (fig. 2). In contrast, isoproterenol increased force of contraction in a concentration-dependent manner; the increase reached 480% at 1 μM (n = 5). CGP 48506 10 μM, 30 μM and 100 μM increased force of contraction by 32%, 113% and 220% of the predrug value, respectively (n = 7). In the same preparations, CGP 48506 concentration-dependently increased time to peak tension, time of relaxation and total contraction time. For instance, CGP 48506 at 10 μM, 30 μM and 100 μM increased total contraction time from 110 ± 3.7 ms to 141 ± 5.3, 173 ± 6.1 and 245 ± 8.0 ms, respectively (n = 7, P < .05). LS at 100 μM, the highest concentration studied, markedly reduced force of contraction to about 35% of the predrug value. LS did not affect the time course of isometric contraction in isolated, electrically driven left atria (data not shown). Similar effects of LS on inotropy and time parameters were observed in left atria from nonreserpinized animals (data not shown).

Contractile studies in papillary muscles. LS exerted a positive inotropic effect similar to that in atria (fig. 3A; for original recording see fig. 4). The positive inotropic effect reached the maximum at 10 μM (130% ± 8.6% of the predrug value, n = 6) and was stable up to 30 min. Isoproterenol increased force to about 330% at the highest effective concentration studied (see fig. 3B). As in the left atria, the force of contraction was markedly reduced by 100 μM LS (to 20% ± 4% of the predrug value, n = 6). Time parameters of contraction in papillary muscles were not affected by LS (data not shown). Similar results for force of contraction and time parameters were obtained in papillary muscles from guinea pigs that had not been pretreated with reserpine (data not shown). The participation of an indirect sympathomimetic action in the positive inotropic effect of LS is hence unlikely. In contrast, CGP 48506 at 30 μM and 100 μM increased force of contraction by 135% and 229% of the predrug value, respectively (n = 5). In order to look in a functional way for possible PDE-inhibitory actions of LS, we preincubated papillary muscles for 30 min with 0.1 μM LS and then applied isoproterenol cumulatively. In control experiments, first solvent (control) was added for 30 min and then isoproterenol. Under these conditions, the EC50 value for the positive inotropic effect of isoproterenol was altered from 15.5 nM to 5.9 nM, as depicted in figure 3B.

Effects of carbachol. A functional way to investigate the involvement of cAMP increase in the positive inotropic action is to study the effect of carbachol on force of contraction in the presence of LS (Endoh and Motomura, 1979). We found that 10 μM carbachol diminished the positive inotropic effect of 10 μM LS by 69.6% ± 6.2% (n = 5, fig. 4). Hence the positive inotropic effect of LS is at least in part mediated via cAMP increase. In contrast, the positive inotropic effect of 10 nM isoproterenol was completely abolished by 10 μM carbachol...
**Measurement of protein phosphorylation.** Because the positive inotropic effects of cAMP-increasing agents are due to phosphorylation of cardiac regulatory proteins, the effects of LS on the phosphorylation pattern in isolated GPVC were studied. Therefore, PLB, Tnl and CP have been identified by pharmacological and immunological criteria as described previously by Neumann et al. (1994, 1995a) and Zimmermann et al. (1995). In isolated GPVC, LS altered the phosphorylation state of PLB and Tnl. Additionally, effects of LS on the phosphorylation state of MLC were investigated. Moreover, by using 5% polyacrylamide separating gels, we could resolve CP in GPVC (Neumann et al., 1995a; 1995b). Results of phosphorylation experiments are summarized in figure 5. LS increased the phosphorylation state of PLB (fig. 5A) and Tnl (fig. 5B) with high potency, starting at 1 nM for PLB and at 10 nM for Tnl. The maximal effect for both was reached at 1 μM and amounted to 134% ± 8.6% of control for PLB and to 124% ± 4.2% of control for Tnl. As shown in figure 5C, the effect of LS on phosphorylation of CP started at 1 μM and amounted to 121% ± 8.0% of control. The phosphorylation state of MLC was not affected by LS in the concentration range 0.1 nM to 10 μM. The phosphorylation state of all proteins studied was significantly decreased at 100 μM LS. Isoproterenol (10 μM) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100 μM) increased PLB phosphorylation by 76% and 69%, respectively (n = 8, each). CGP 48506 (100 μM) did not affect protein phosphorylation (Zimmermann et al., 1996a).

### Calcium currents.

To understand further the molecular mechanism of action of LS, we performed electrophysiological experiments. Thus the influence of LS on the amplitude of L-type Ca$^{2+}$ current in guinea pig ventricular cardiomyocytes was studied. A typical experiment illustrating original current traces and the time course of the amplitude of the L-type Ca$^{2+}$ current is depicted in figure 6. Original current traces (panel A) correspond to the time-points indicated in the lower trace (panel B). At the maximally effective positive inotropic concentration, LS (10 μM) increased the amplitude by 0.2 pmol/mg protein, though this concentration exerts strong negative inotropic effects and reduces the phosphorylation state of cardiac regulatory proteins. 3-Isobutyl-1-methylxanthine (100 μM) and isoproterenol (10 μM) increased cAMP content to 12.8 ± 0.74 (n = 6) pmol/mg protein and 12.4 ± 0.63 pmol/mg protein (n = 7), respectively, in guinea pig ventricular cardiomyocytes. However, CGP 48506 (100 μM) in parallel experiments did not affect cAMP content (Zimmermann et al., 1996a).

### Levels of cAMP.

Furthermore, the effects of LS on cAMP content were directly measured (table 1). LS (1 μM, 30 min) increased cAMP content from 6.3 ± 0.33 to 8.1 ± 0.35 pmol/mg protein in guinea pig ventricular cardiomyocytes. LS at 100 μM increased cAMP content even further (to 9.3 ± 0.24 pmol/mg protein) (data not shown), and the positive inotropic effects of 10 μM, 30 μM and 100 μM CGP 48506 were not affected by carbachol (Zimmermann et al., 1996a).

### Table 1

**Effects of levosimendan on cAMP content in guinea pig ventricular cardiomyocytes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP Content (pmol/mg protein)</th>
<th>Number of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr</td>
<td>6.3 ± 0.33</td>
<td>10</td>
</tr>
<tr>
<td>LS 0.01 μM</td>
<td>5.9 ± 0.28</td>
<td>10</td>
</tr>
<tr>
<td>LS 1 μM</td>
<td>8.1 ± 0.35*</td>
<td>10</td>
</tr>
<tr>
<td>LS 100 μM</td>
<td>9.3 ± 0.19*</td>
<td>10</td>
</tr>
</tbody>
</table>

* P < .05 vs. control (Ctr).
of L-type Ca\(^{++}\) current to 402% ± 86% of control (n = 4). The effect was not completely reversible upon washout up to 15 min. For comparison, the effects of the beta adrenoceptor agonist isoproterenol were studied. In the same experimental setting, isoproterenol (10 nM) enhanced the amplitude of L-type Ca\(^{++}\)-current to 485% ± 75% of control (n = 10).

Discussion

In the present work, we wanted to address the question of whether levosimendan has a direct positive inotropic effect that is due either to phosphodiesterase inhibition (increase in cAMP levels) or to calcium sensitization. In recent years, many phosphodiesterase inhibitors, such as sulmazole, isomazole, adibendan and EMD 53998 (for review see Rüegg and Solaro, 1993; Lee and Allen, 1995), have been reported to possess calcium-sensitizing activities. One can distinguish 1) pure phosphodiesterase inhibitors devoid of calcium-sensitizing activities (3-isobutyl-1-methyl-xanthine), 2) drugs that are mainly phosphodiesterase inhibitors with ancillary calcium-sensitizing properties (pimobendan, EMD 57033) and 3) drugs that are calcium sensitizers devoid of phosphodiesterase inhibitory activities (CGP 48506).

LS was discovered by screening for troponin C binding compounds. Binding of LS to troponin C is thought to lead to its calcium-sensitizing properties (Haikala et al., 1995). In addition, LS potently (IC\(_{50}\) = 25 mM) inhibited phosphodiesterase type III, the isoenzyme assumed to be most relevant for the positive inotropic action of phosphodiesterase inhibitors (Raasmaja et al., 1991).

From the literature, it was unclear whether LS is actually a directly acting positive inotropic drug. An indirect positive inotropic effect by 1) release of catecholamines, 2) vasodilatory effects and 3) alteration in the heart rate could not be completely ruled out. For instance, Rump et al. (1994), Edes et al. (1995) and Haikala et al. (1995) have investigated the cardiac effects of LS in preparations from nonreserpinized animals. The pretreatment with reserpine makes it possible to exclude indirect sympatheticeffects. In addition, Rump et al. (1994) have studied effects of LS in whole rabbit hearts perfused at constant pressure. In this experimental setting, an increase in force of contraction resulting from coronary vasodilation (as shown for LS by Végh et al., 1992) cannot be distinguished from direct effects on the contractile apparatus. Moreover, Edes et al. (1995) have investigated effects of LS in spontaneously beating guinea pig hearts. Thus the authors could not differentiate between a direct positive inotropic effect and an indirect positive inotropic effect due to enhanced rate of contraction (treppe phenomenon).

The findings of the present work with tissue from reserpinized animals strongly suggest that LS directly increased force of contraction. However, the maximal positive inotropic effect was smaller than that of isoproterenol, CGP 48506 (this study; Zimmermann et al., 1996a) or, for instance, EMD 57033 (Lues et al., 1993; Neumann et al., 1995c).
Some findings of this report are compatible with the view that LS is a calcium sensitizer. For instance, LS did not alter time parameters of contraction in isolated preparations from the guinea pig heart (left atria and papillary muscle). Compounds that act solely by an increase in cAMP (such as isoproterenol) classically decrease time to peak tension, time of relaxation and total contraction time. However, EMD 57033 strongly prolongs time parameters (Luès et al., 1993; Neumann et al., 1995c). Likewise, CGP 48506 prolongs time parameters in atria (this study) and papillary muscles (Zimmermann et al., 1996a). Hence LS probably is not a potent calcium sensitizer and more resembles pimobendan, which barely affects time parameters. One might argue that the effects of LS as PDE inhibitor (see below) and as calcium sensitizer balance each other.

We present several lines of evidence that LS acts to a certain extent *via* an increase in cAMP. For instance, carbachol diminished the positive inotropic effect of EMD 57033 (Neumann et al., 1995c), pimobendan (Brunkhorst et al., 1989), LS (this study) and isoproterenol. In contrast, carbachol failed to decrease the positive inotropic effect of CGP 48506 (Zimmermann et al., 1996a). Furthermore, LS potentiated the positive inotropic effect of isoproterenol, which is typical of PDE inhibitors (such as 3-isobutyl-1-methylxanthine; Brückner et al., 1980). In addition, the positive chronotropic action observed in isolated, spontaneously beating right atria in the presence of LS is typical of cAMP-increasing agents (such as isoproterenol and 3-isobutyl-1-methylxanthine; Brunkhorst et al., 1989), and it is not observed with CGP 48506.

Extending the work of Edes et al. (1995, multicellular preparations, *i.e.*, whole hearts) to the cardiomyocyte level, we have demonstrated that LS, like isoproterenol and the PDE inhibitor 3-isobutyl-1-methylxanthine, elevated cAMP content in isolated ventricular cardiomyocytes. In contrast, CGP 48506 does not affect the cardiac cAMP content (Zimmermann et al., 1996a). LS markedly enhanced the amplitude of l-type Ca$^{2+}$ current in a way similar to a cAMP-elevating drug such as isoproterenol. Because it is known that the electrophysiological effects of isoproterenol are due to cAMP-dependent phosphorylation (Hartzell et al., 1991), this finding is also consistent with the hypothesis that LS may act *via* an increase in cAMP.

Measuring the phosphorylation of proteins in isolated cardiomyocytes, we achieved results similar to those reported by Edes et al. (1995) on mixed cellular preparations (whole hearts). We focused on PLB, Tnl and CP. These regulatory proteins are phosphorylated by cAMP-dependent protein kinase, and the positive inotropic and positive lusitropic effects of cAMP-increasing agents have been attributed at least in part to their phosphorylation (Rapundalo et al., 1989; Hofmann et al., 1991; Sham et al., 1991; Luo et al., 1994). LS (less than 100 μM) increased the phosphorylation state of PLB, Tnl and CP. However, an increase in the phosphorylation state of PLB and Tnl by LS should hasten relaxation, and this was not observed. The discrepancy in the present work between increased phosphorylation and unchanged time of relaxation could be explained by a Ca$^{2+}$-sensitizing effect of LS. This explanation is supported by our finding that EMD 57033 increased the phosphorylation state of PLB and Tnl even while prolonging the duration of contraction (Neumann et al., 1995c). In contrast, CGP 48506, which is devoid of PDE inhibitory properties (Herold et al., 1995; Neumann et al., 1996; Zimmermann et al., 1996a) does not enhance protein phosphorylation.

The marked decrease induced by 100 μM LS in the phosphorylation state of all phosphoproteins studied does not result from a reduction in cAMP content but could explain the negative inotropic effect of 100 μM LS. One can speculate that toxic concentrations of LS might activate protein phosphatases (Zimmermann et al., 1996b) or inhibit the cAMP-dependent protein kinase (for milrinone see Earl et al., 1986).

In summary, we characterized the mechanism of action of LS in the mammalian heart. We suggest that the positive inotropic effects of LS are due at least in part to an increase in cAMP. The marked decrease in PLB and Tnl phosphorylation at toxic concentrations of LS may act via a reduction in cAMP. This link is based on the facts that 1) LS increases the rate of contraction, 2) LS increases the amplitude of the l-type Ca$^{2+}$ current, 3) the positive inotropic effect of LS is attenuated by carbachol, 4) LS potentiates the positive inotropic response to isoproterenol, 5) LS increases the phosphorylation of cardiac regulatory proteins and 6) LS increases tissue levels of cAMP.

**Acknowledgments**

We gratefully acknowledge the excellent technical assistance of Mrs. Cordula Vischedyck.

**Fig. 6.** Effects of LS on the l-type Ca$^{2+}$ current in isolated guinea pig ventricular cardiomyocytes. A) Original traces demonstrate the effect of LS (10 μM) on the amplitude of l-type Ca$^{2+}$ current. Original tracings in figure 6A were obtained at time-points 1 and 2 as indicated by arrows in figure 6B. Point 1 is shortly before application of LS, and point 2 is when the effect on the current was maximal (less than 10 min). B) The time course for the effect of LS (10 μM) on the amplitude of l-type Ca$^{2+}$ current of a guinea pig ventricular myocyte. The current was elicited by voltage steps from −40 mV as holding potential to +10 mV for 300 ms at a frequency of 0.1 Hz.