Effects of inhibitors of protein kinase C and Na^+/H^+ exchange on α_1 -adrenoceptor-mediated inotropic responses in the rat left ventricular papillary muscle

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- 1 α_1 -Adrenoceptor stimulation of rat left ventricular papillary muscle produced a triphasic inotropic response: an initial transient positive inotropic effect (PIE) followed by a transient negative inotropic effect (NIE) and a sustained PIE.
- 2 The protein kinase C inhibitor, staurosporine, at concentrations ranging from 30 nM to 100 nM inhibited the sustained PIE, but had no significant effect on the transient PIE and NIE.
- 3 H-7, 1-(5-isoquinoline sulphonyl)-2-methylpiperazine, a less specific inhibitor of protein kinase C than staurosporine, at a concentration of 100 μM inhibited both the transient NIE and the sustained PIE without affecting the transient PIE.
- 4 Amiloride, an inhibitor of Na^+/H^+ exchange, at concentrations ranging from 0.1 mM to 1 mM inhibited the sustained PIE and, at higher concentrations, also inhibited the transient NIE.
- 5 An amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), inhibited only the sustained PIE with an IC_{50} of 0.3 μM which is approximately two orders of magnitude lower than amiloride.
- 6 The receptor-linked stimulation of Na^+/H^+ exchange through protein kinase C activation may be a mechanism for α_1 -adrenoceptor-mediated sustained PIE.

Introduction

The mechanism of α_1 -adrenoceptor-mediated inotropic responses has been a matter of debate for many years (Ahlquist, 1948; Endoh *et al.*, 1982). A growing body of evidence indicates that an α_1 -adrenoceptor-mediated positive inotropic effect (PIE) is associated with the receptor-linked degradation of phosphatidyl-inositol 4,5-bisphosphate and the resulting generation of inositol trisphosphate (IP_3) and diacylglycerol (Otani *et al.*, 1988; Scholz *et al.*, 1988). IP_3 is known to mobilize Ca^{2+} from intracellular compartments and is thought to participate as a transient component of the cellular response, whereas diacylglycerol may produce a sustained phase of the cellular response via the activation of protein kinase C (Berridge, 1984; Williamson *et al.*, 1985; Howe *et al.*, 1986). α_1 -Adrenoceptor stimulation with phenylephrine in rat isolated left ventricular papillary muscle produces a triphasic inotropic response, in which IP_3 -induced Ca^{2+} -mobilization has been shown to be involved in the initial transient PIE and the following transient negative inotropic effect (NIE) (Otani *et al.*, 1988). However, the mechanism involved in the phenylephrine-induced sustained PIE has not been well analysed. In this study, we have examined the role of protein kinase C activation in the sustained PIE by use of inhibitors of the kinase: staurosporine and 1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7). The effects of these two protein kinase C inhibitors were tested because the mechanism of action and potency of protein kinase C inhibition are different (Nakadate *et al.*, 1988). Further, participation of the Na^+/H^+ exchange system in the sustained PIE was also examined by use of inhibitors of the Na^+/H^+ exchange system: amiloride and 5-(N-methyl-N-isobutyl)amiloride (MIBA). Activation of protein kinase C is known to stimulate a Na^+/H^+ exchange system in a number of different cell types including cardiac cells (Vigne *et al.*, 1985; Griendling *et al.*, 1988; Siffert & Akkerman, 1988). In addition, this antiporter could increase

contractility in cardiac muscle by increasing Ca^{2+} influx through $\text{Na}^+/\text{Ca}^{2+}$ exchange, following an accumulation of intracellular Na^+ (Ikeda *et al.*, 1988), or by increasing the sensitivity of the contractile protein for Ca^{2+} through intracellular alkalinization (Fabiato & Fabiato, 1978).

Methods

Male Sprague-Dawley rats weighing 250–300 g were anaesthetized with an intra-peritoneal injection of sodium pentobarbitone, and the heart was quickly removed. Anterior left ventricular papillary muscles of diameter 0.8–1.0 mm were suspended in organ baths containing Tyrode solution of the following composition (mM): NaCl 122.5, KCl 5.4, CaCl_2 1.8, MgCl_2 1.1, NaHCO_3 24 and glucose 10 (pH 7.4) aerated with 95% O_2 and 5% CO_2 at 32°C. The muscle was initially loaded with 500 mg and driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 ms and a voltage ranging from 2–4 V which was twice threshold. The isometric tension was measured by a force displacement transducer (Shinko-Tsusin, Japan. UL-2) and recorded on a pen-writing recorder (Seconic, Japan. SS-250F) through an amplifier (Shinko-Tsusin, DS-601B). After 50 min equilibration, experimentation was started. The preparation was treated with 0.3 μM propranolol for 10 min and exposed to 10 μM phenylephrine. This concentration of phenylephrine was shown to produce a submaximum inotropic response, and propranolol was required to inhibit β -adrenoceptor stimulation at this phenylephrine concentration (Otani *et al.*, 1986). A single dose of H-7, staurosporine, amiloride or its analogue was added to the buffer 30 min before the addition of phenylephrine, and only a single response to phenylephrine was tested in each tissue to avoid the possible occurrence of tachyphylaxis. Changes in contractility following the administration of phenylephrine were measured when the peak responses appeared, respectively, and expressed as a % of that obtained just before the addition of phenylephrine.

1-(5-Isoquinoline sulphonyl)-2-methylpiperazine (H-7) and staurosporine were obtained from Seikagaku-Kogyo Co., Ltd.,

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Japan and Kyowa-medex Co., Ltd., Japan, respectively. The amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA) was synthesized by a previously described method (Cragoe *et al.*, 1967). Prazosin was a gift from Pfizer Inc., New York. Phenylephrine and amiloride were purchased from Sigma Chemical Co., St. Louis, Mo. Staurosporine and MIBA were prepared as stock solutions in DMSO (dimethyl sulphoxide). Final DMSO concentrations in bath media did not exceed 0.01%, and this concentration of DMSO was used as a vehicle control.

Statistical analysis

The results are expressed as mean $\pm$ s.e.mean. Student's *t* test was used for statistical analysis. The differences with $P < 0.05$ were considered to be significant.

Results

Treatment of rat left ventricular papillary muscle with $10\ \mu\text{M}$ phenylephrine in the presence of $0.3\ \mu\text{M}$ propranolol produced a triphasic inotropic response: a transient PIE (phase I) followed by a transient NIE (phase II) and a sustained PIE (phase III) (Figure 1a). An α_1 -adrenoceptor antagonist, prazosin, at concentrations ranging from 0.01 to $1\ \mu\text{M}$ concentration-dependently inhibited all the phases of the inotropic response (Figure 1b).

As shown in Figure 2, the protein kinase C inhibitors staurosporine ($100\ \text{nm}$) and H-7 ($100\ \mu\text{M}$) reduced the sustained

Figure 1 Effects of prazosin on α_1 -adrenoceptor-mediated triphasic inotropic response in rat left ventricular papillary muscle. (a) Inotropic response induced by $10\ \mu\text{M}$ phenylephrine (PE) in the presence of $0.3\ \mu\text{M}$ propranolol (Prop). The scale shown in the graph indicates the active contractile force. I, transient positive inotropic response; II, transient negative inotropic response; III, sustained positive inotropic response. (b) Effect of pretreatment with 0.001– $1\ \mu\text{M}$ prazosin for 30 min on PE-induced triphasic inotropic responses in the presence of $0.3\ \mu\text{M}$ propranolol. The change in the contractile force was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represents the mean of 6 preparations; vertical lines show s.e.mean. * $P < 0.05$, ** $P < 0.01$ compared to the value without drug treatment.

Figure 2 Representative traces of effects of staurosporine and H-7 on α_1 -adrenoceptor-mediated inotropic responses. Rat left ventricular papillary muscle treated with vehicle (0.01% dimethyl sulphoxide, a), $100\ \text{nm}$ staurosporine (St, b), or $100\ \mu\text{M}$ H-7 (c) for 30 min was exposed to $10\ \mu\text{M}$ phenylephrine (PE) in the presence of $0.3\ \mu\text{M}$ propranolol (Prop).

PIE. Treatment with staurosporine ($100\ \text{nm}$) or H-7 ($100\ \mu\text{M}$) for 30 min reduced the basal contractility to $76.3 \pm 4.5\%$ (mean $\pm$ s.e.mean; $n = 6$) or $89.2 \pm 2.0\%$ (mean $\pm$ s.e.mean; $n = 6$), respectively, of that observed before the treatment. The effects of different concentrations of these reagents on each phase of the triphasic inotropic response are summarized in Figure 3. Staurosporine at concentrations ranging from 30 to $100\ \text{nm}$ inhibited the sustained PIE in a concentration-dependent manner, but had no effect on the transient PIE. The transient NIE tended to be slightly augmented by $100\ \text{nm}$ staurosporine (Figure 3a). H-7 at a concentration of $100\ \mu\text{M}$ also inhibited the sustained PIE, though less potently than staurosporine, with no effect on the transient PIE. However, this protein kinase C inhibitor reduced the transient NIE in a concentration-dependent manner (Figure 3b).

The effects of Na^+/H^+ exchange inhibitors, amiloride ($1\ \text{mM}$) and its analogue MIBA ($100\ \text{nM}$), on the phenylephrine-induced inotropic responses are shown in representative traces (Figure 4). Both inhibitors markedly reduced the sustained PIE. Basal contractility did not change significantly on treatment with $1\ \text{mM}$ amiloride, but it was reduced by $100\ \mu\text{M}$

Figure 3 Effects of staurosporine and H-7 on α_1 -adrenoceptor-mediated inotropic response in rat left ventricular papillary muscle. (a) Concentration-response effect of staurosporine on the $10\ \mu\text{M}$ phenylephrine (PE)-induced inotropic response. (b) Concentration-response effect of H-7 on the $10\ \mu\text{M}$ PE-induced inotropic response. In each figure, the maximum change in contractile force in each inotropic phase was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represents the mean of 6 preparations; vertical lines show s.e.mean. † $P < 0.1$, * $P < 0.05$, ** $P < 0.01$ compared to the value without drug treatment.

Figure 4 Representative traces of the effects of amiloride and an amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), on the α_1 -adrenoceptor-mediated inotropic response. Rat left ventricular papillary muscle treated with vehicle (0.01% dimethyl sulphoxide, a), 1 mM amiloride (b), or 100 μ M MIBA (c) for 30 min was exposed to 10 μ M phenylephrine (PE) in the presence of 0.3 μ M propranolol (Prop).

MIBA to $72.5 \pm 5.8\%$ (mean $\pm$ s.e.mean; $n = 6$) of that observed before the treatment with MIBA. The effects of different concentrations of these inhibitors on each contractile phase are summarized in Figure 5. Amiloride at concentrations ranging from 0.1 to 1 mM inhibited the sustained PIE, and also inhibited the transient NIE at and above 0.3 mM (Figure 5a). A more specific inhibitor for Na^+/H^+ exchange, MIBA, inhibited only the sustained PIE with a potency approximately two orders of magnitude greater than amiloride (Figure 5b).

Discussion

The present study evaluated the concentration-response effects of an α_1 -adrenoceptor blocker and inhibitors of protein kinase C and Na^+/H^+ exchange, to determine whether protein kinase C and Na^+/H^+ exchange activity are involved in the sustained PIE mediated by α_1 -adrenoceptor stimulation. α_1 -Adrenoceptor stimulation provoked a triphasic inotropic response, as has been observed in a previous study (Otani *et al.*, 1988). Prazosin showed a similar concentration-dependent

inhibition of the triphasic inotropic response, suggesting that all of these inotropic responses are mediated through α_1 -adrenoceptor stimulation.

Both staurosporine and H-7 inhibited the sustained PIE without affecting the transient PIE, but the effect of these protein kinase C inhibitors on the transient NIE was opposite: staurosporine augmented the NIE, and H-7 inhibited it. A common inhibitory effect of staurosporine and H-7 on the sustained PIE and opposite effects of the transient NIE support the contention that protein kinase C is involved in the phenylephrine-induced sustained PIE, but not in the transient inotropic responses. Although the reason for the differential effect of H-7 on transient NIE from that observed with staurosporine is currently unknown, it may be related to their diverse action on some other cellular mechanisms.

Activation of protein kinase C could lead to a sustained PIE in cardiac muscle via Ca^{2+} channel activation and/or via stimulation of Na^+/H^+ exchange (Dösemeci *et al.*, 1988; Ikeda *et al.*, 1988). However, the role of the slow inward current in producing PIE is still controversial (Dösemeci *et al.*, 1988). On the other hand, the present study showed that the Na^+/H^+ exchange inhibitors amiloride and MIBA inhibited the α_1 -adrenoceptor-mediated inotropic response. The amiloride analogue, MIBA, was used as a specific inhibitor of Na^+/H^+ exchange, with a lower K_i of 0.44 μ M compared with the K_i of 84 μ M for amiloride (Kleyman & Cragoe, 1988). Since these two Na^+/H^+ exchange inhibitors inhibited the sustained PIE but had no or opposite effects on the transient PIE or NIE, it is reasonable to assume that stimulation of Na^+/H^+ exchange is involved in the sustained PIE, but not in the transient inotropic responses. Although the mechanism by which Na^+/H^+ exchange induced the sustained PIE was not determined in this study, at least two mechanisms are envisaged. Stimulation of Na^+/H^+ exchange operates toward the stimulation of Na^+ influx, which may in turn increase intracellular Ca^{2+} via $\text{Na}^+/\text{Ca}^{2+}$ exchange (Frelin *et al.*, 1984; Ikeda *et al.*, 1988). Further, intracellular alkalization provoked by Na^+/H^+ exchange may increase the sensitivity of myofilaments for Ca^{2+} (Fabiato & Fabiato, 1978). Either mechanism could result in an increase in the force of contraction.

The inhibition of the transient NIE by amiloride at higher concentrations may be accounted for by the inhibition of Ca^{2+} extrusion via $\text{Na}^+/\text{Ca}^{2+}$ exchange, since amiloride acts as an inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchange with a K_i of 1 mM in the cardiac sarcolemmal vesicle (Siegel *et al.*, 1984), and the transient NIE is thought to be at least in part due to stimulated Ca^{2+} extrusion through $\text{Na}^+/\text{Ca}^{2+}$ exchange. Furthermore, this assumption raises the possibility that H-7 and amiloride, both of which inhibited the transient NIE, share a common mechanism of action in preventing the loss of intracellular Ca^{2+} during a period of transient NIE.

Amiloride is also known to act as an α_1 -adrenoceptor antagonist at a concentration range similar to that inhibiting Na^+/H^+ exchange in rat perfused liver (Haüssinger *et al.*, 1987). α_1 -Adrenoceptor antagonism by amiloride, however, is unlikely to provide an explanation for the inhibition of the transient NIE and the sustained PIE, because amiloride failed to inhibit the transient PIE, which has been shown to correlate closely with an α_1 -adrenoceptor-mediated increase in IP_3 (Otani *et al.*, 1988). Prazosin inhibition of the transient PIE is consistent with this notion.

In summary, this paper provides evidence for the first time that α_1 -adrenoceptor-mediated sustained PIE is sensitive to inhibitors of Na^+/H^+ exchange as well as those of protein kinase C. Receptor-linked stimulation of Na^+/H^+ exchange through protein kinase C activation is strongly suggested as a mechanism for the sustained PIE in the α_1 -adrenoceptor-mediated inotropic response.

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan, and also by a grant from Kansai Medical University.

Figure 5 Effects of amiloride and an amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), on the α_1 -adrenoceptor-mediated inotropic response in rat left ventricular papillary muscle. (a) Concentration-response effects of amiloride on 10 μ M phenylephrine (PE)-induced inotropic responses. (b) Concentration-response effects of MIBA on the 10 μ M PE-induced inotropic response. In (a) and (b), the maximum change in contractile force in each inotropic phase was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represents the mean of 6 preparations and vertical lines indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$ compared to the value without drug treatment.

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(Received July 17, 1989

Revised January 3, 1990

Accepted January 16, 1990)