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Possible Involvement of Protein Kinase C in the Modulation of Inotropic and Chronotropic Effects Induced by Ouabain in Rat Right Atrial Muscles

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Abstract

Modulations of the inotropic and chronotropic effects of ouabain and protein kinase C (PKC) stimulation with phorbol esters in rat right atria were examined. Cumulative administration of ouabain (3–30 μM) caused a positive inotropic effect in a concentration-dependent manner, but did not produce a chronotropic effect. A single administration of ouabain (30 μM) also had similar effects: $+74{\cdot}4 \pm 8{\cdot}4\%$ ($n=23$, $P<0{\cdot}01$) in the contractile force and $-0{\cdot}7 \pm 1{\cdot}3\%$ ($n=23$, $P>0{\cdot}05$) in the sinus rate. Addition of phorbol esters reinforced the ouabain-evoked positive inotropic effect: $26{\cdot}5 \pm 8{\cdot}9\%$ ($n=6$, $P<0{\cdot}05$) with 100 μM 4-β-phorbol-12,13-dibutyrate (PDB), and $6{\cdot}4 \pm 3{\cdot}3\%$ ($n=6$, $P>0{\cdot}05$) with 100 μM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Simultaneously, the mixture of ouabain and phorbol ester raised the resting tension. Phorbol esters alone caused a positive inotropic effect (by about 21–27%). Non-PKC activating phorbol ester, 4-α-phorbol-12,13-didecanoate (PDD, 100 μM), did not have any effect. Pretreatment with the PKC inhibitor (staurosporine 100 μM) significantly decreased the ouabain-induced positive inotropic effect and caused a negative chronotropic effect, but H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride) (5 μM) had no effect.

These results suggest that PKC stimulation may be involved in the ouabain-evoked responses in the right atria of rat as seen by increased cellular Ca^{2+} concentration (and Ca^{2+}-sensitivity); thus the positive inotropic effect may not be due only to modulation of Na^+/K^+ pump activity.

In clinical use, cardiac glycosides have a positive inotropic effect, a negative chronotropic effect and a negative dromotropic effect. The positive inotropic effect is known to be produced by Na^+/K^+ pump blockade by ATPase inhibition (Akera & Brody 1978), stimulation of Ca^{2+} current (I_{Ca}) (Lederer & Eisner 1982; Marban & Tsien 1982), and Ca^{2+} release from sarcoplasmic reticulum (Schiebinger & Cragoe 1993). However, Satoh (1994a) and Josephson & Sperelakis (1977) showed that ouabain never stimulates L-type Ca^{2+} current (I_{Ca}), but inhibits it. In addition, strophanthidine at lower concentrations (1–10 nM) enhances the contractile force in spite of stimulation of the Na^+/K^+ ATPase activity (Peters et al 1974). Thus, the positive effect is not necessarily correlated with the I_{Ca} stimulation and pump inhibition (Rhee et al 1976). Thus, the multiple actions of ouabain might be produced by the other mechanisms.

For the contractile force of cardiac muscles, protein kinase A (PKA), dependent on cAMP, and protein kinase G (PK-G), dependent on cGMP, are major regulators of the contractile force. Furthermore, inositol trisphosphate (IP_3) and diacylglycerol, via phosphatidylinositol turnover, are also associated with the contraction (Berridge & Irvine 1984). PKC activated by diacylglycerol plays an important role in the phosphorylation of many proteins (Nishizuka 1984). PKC stimulation by phorbol esters (tumour promoters) cause the negative chronotropic and inotropic effects in rabbit sino-atrial (SA) nodal cells (Satoh & Hashimoto 1988), in young embryonic chick ventricular cells (Satoh 1995) and in canine Purkinje fibers (Satoh et al 1992). The I_{Ca} and delayed rectifier K^+ current (I_K) are inhibited (Satoh & Hashimoto 1988; Satoh 1992, 1995). However, PKC elevates the cellular Ca^{2+} concentration ($[Ca]_i$) in rabbit SA nodal cells, and elicits arrhythmias (Satoh & Hashimoto 1988; Satoh 1994b). Most recently, PKC stimulation has also been reported to enhance the contraction due to an increase in sensitivity to Ca^{2+} of the contractile proteins in vascular smooth cells (Jiang & Morgan 1987; Nishimura et al 1990).

Thus, PKC stimulation regulates the contraction of cardiac muscles, and might be involved with the positive inotropic effect induced by ouabain. In the present work, therefore, we investigated the possibility for its involvement. We examined whether PKC is associated with the ouabain-evoked positive inotropic effect, and how the PKC stimulation modulates the effects of ouabain.

Materials and Methods

Wistar rats (male) of 6- to 12-weeks-old, weighing 250–450 g, were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.p.). The detail of the methods has been described in our recent reports (Nakatani et al 1994). In brief, the heart was quickly excised. Isometric tension was measured using a force displacement transducer (Nihon Kohden, TB-652T, Tokyo, Japan). The contractile force and sinus rate were recorded on a thermal recorder (Nihon Kohden, WS-641G).

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Solutions

The composition of modified Tyrode solution (mM) was as follows: 137 NaCl, 2·7 KCl, 1·8 $CaCl_2$, 1·0 $MgCl_2$, 11·9 $NaHCO_3$, 0·45 NaH_2PO_4, and 5·5 glucose. The pH was adjusted to 7·4 with NaOH. The bath solution was oxygenated by 95% O_2 and 5% CO_2. All experiments were performed at 36°C.

Drugs used were ouabain (Sigma Chemical Co., St Louis, MO), 4-β-phorbol-12,13-dibutyrate (PDB), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 4-α-phorbol-12,13-didecanoate (PDD). H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride) (Seikagaku Kogyo Co., Tokyo) and staurosporine (Wako Pure Chemical Ind., Osaka, Japan) were also used. Phorbol esters were dissolved with dimethyl sulphoxide (DMSO) and were then adjusted to the desired concentrations directly in the bath solution.

Statistical analysis

Values are given as mean ± s.e.m. The comparisons were analysed by analysis of variance and Student's *t*-test. Probability levels of less than 0·05 were considered significantly different.

Results

Ouabain at 3 to 30 μM increased the contractile force in a concentration-dependent manner, but did not affect the sinus rate (Fig. 1A). Ouabain was cumulatively added to the bath solution. The averaged values are summarized in Fig. 1C. The positive inotropic effect was 29·2 ± 6·2% (n = 6, $P < 0.01$) at 10 μM, and 69·7 ± 14·8% (n = 5, $P < 0.05$) at 30 μM. On the other hand, a single administration of 30 μM ouabain also caused a positive inotropic effect by 74·4 ± 8·4% (n = 23, $P < 0.01$)(Fig. 1B). No significant difference between the cumulative and single administrations was observed.

Addition of phorbol esters to ouabain (30 μM)-containing solution was carried out to examine the modulation of contractile force (Fig. 2A-C). PDB at 30 nM reinforced the positive inotropic effect induced by 30 μM ouabain by 10·1 ± 5·7% (n = 6, $P > 0.05$); PDB at 100 nM reinforced the effect by 26·5 ± 8·9% (n = 6, $P < 0.05$). PDB at 30 nM also increased the positive chronotropic effect by 8·3 ± 4·2% (n = 6, $P > 0.05$) and at 100 nM by 23·0 ± 7·3% (n = 6, $P < 0.05$). On the other hand, TPA (10-100 nM) also increased the positive inotropic and chronotropic effects, but not to any significant extent (at 100 nM the increase was 6·4 ± 3·3%, n = 6 and 3·7 ± 2·2%, n = 6, respectively). Simultaneously, PDB at 100 nM significantly raised the resting tension by 780 ± 11 mg (n = 6), but TPA at 100 nM had less or no effect. On the other hand, phorbol esters (10-100 nM) alone had a positive inotropic effect in a concentration-dependent manner: 27·3 ± 6·5% (n = 8, $P < 0.01$) in 100 nM PDB and 21·4 ± 7·2% (n = 8, $P < 0.05$) in 100 nM TPA (Table 1). PDB had more potent actions than TPA. The sinus rate was unaffected by both PDB and TPA. On the other hand, PDD, a non-PKC activating phorbol ester, was added to compare the effects of PKC stimulation. PDD (100 nM) did not affect the inotropic and chronotropic effects induced by ouabain (Fig. 2C).

Both inhibitors of PKC, H-7 and staurosporine, were applied to examine whether the ouabain-evoked responses might be associated with PKC stimulation. In the presence of both inhibitors, ouabain still caused the positive inotropic effect, as shown in Figs 3A and 3B. H-7 (5 μM) alone had negative inotropic and chronotropic effects by 12·7 ± 1·5% (n = 14, $P < 0.001$) and by 2·1 ± 1·5% (n = 14, $P > 0.05$), respectively (Table 2). Staurosporine alone caused changes of − 14·4 ± 3·6% (n = 23, $P < 0.01$) and − 10·2 ± 1·7% (n = 23, $P < 0.001$), respectively. H-7 (5 μM) did not modify

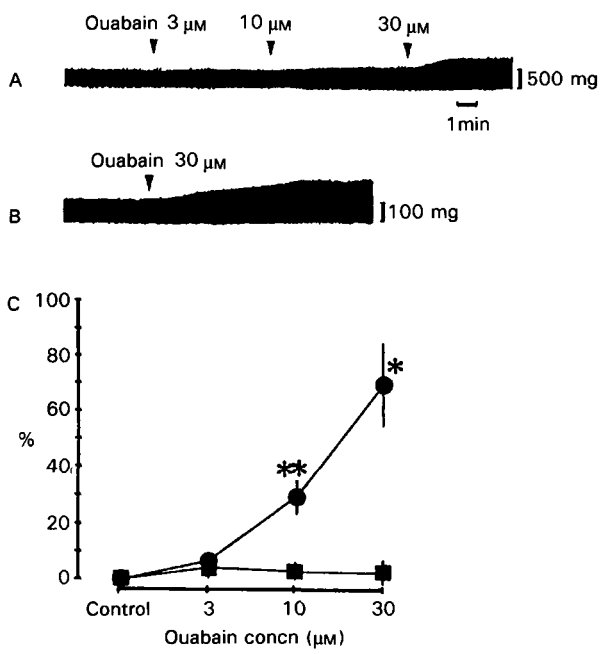

FIG. 1. Effects of ouabain on the contractile force and sinus rate in right atrium of rat. A. Inotropic effect of cumulative administration (3-30 μM) of ouabain. B. Effect of 30 μM ouabain as a single administration. C. Concentration-response curves of the contractile force (●) and sinus rate (■). Values are expressed as mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, with respect to control values.

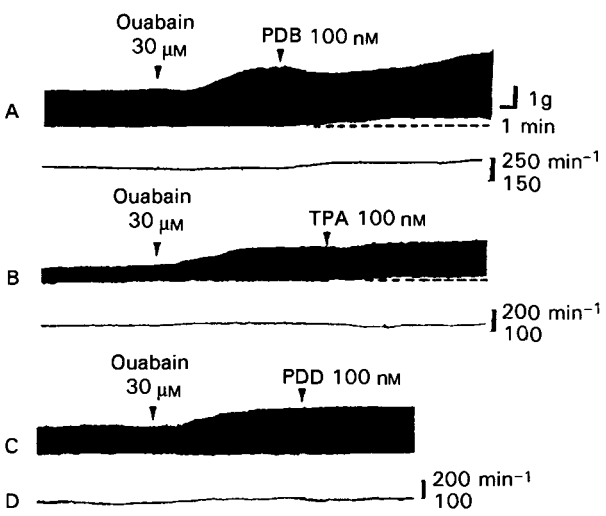

FIG. 2. Modulation of the ouabain-evoked responses by phorbol esters. Initially ouabain (30 μM) was administered and then phorbol ester was added. Dashed line indicates a control level of the resting tension. A. Effect of addition of 100 nM PDB on the contractile force and sinus rate. B. Addition of 100 nM TPA. C. Addition of 100 nM PDD, phorbol ester analogue inactive for PKC stimulation.

Table 1. Effects of PKC inhibitors on the responses induced by phorbol esters in rat right atria.

	n	Contractile force	Sinus rate
PDB 10 nM	8	5·6 ± 3·2	2·2 ± 3·1
PDB 30 nM	8	13·8 ± 3·1*	3·3 ± 4·0
PDB 100 nM	8	27·3 ± 6·5**	7·1 ± 3·6
+ H-7 5 μM	8	− 7·8 ± 2·1	− 0·6 ± 2·5
+ Staurosporine 100 nM	8	− 10·4 ± 2·7*	− 1·8 ± 3·4
TPA 10 nM	8	3·3 ± 1·7	1·2 ± 1·1
TPA 30 nM	8	10·1 ± 3·8*	1·6 ± 1·9
TPA 100 nM	8	21·4 ± 7·2*	2·5 ± 2·8
+ H-7 5 μM	8	− 3·5 ± 3·4	− 5·1 ± 2·8
+ Staurosporine 100 nM	8	− 7·8 ± 2·0*	− 6·9 ± 3·6
PDD 30 nM	7	1·3 ± 1·5	0·8 ± 1·2
PDD 100 nM	7	2·2 ± 3·6	− 4·6 ± 2·3
+ H-7 5 μM	7	− 3·3 ± 2·8	− 2·1 ± 2·0
+ Staurosporine 100 nM	7	− 4·8 ± 3·0	− 4·2 ± 3·4

Values (%) represent mean ± s.e.m. PDB: 4-β-phorbol-12,13-dibutyrate. TPA: 12-*O*-tetradecanoyl-phorbol-13-acetate. PDD: 4-α-phorbol-12,13-didecanoate. *$P < 0.05$, **$P < 0.01$, with respect to control value.

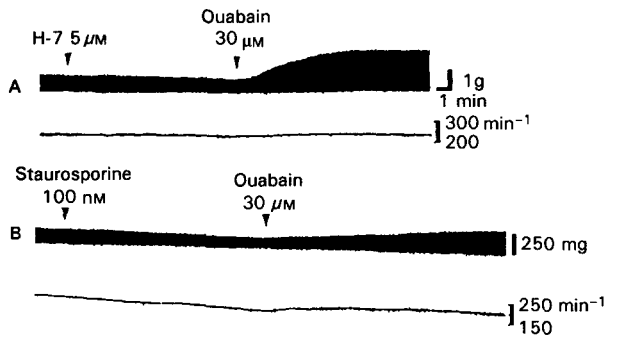

FIG. 3. Modulation of the effects of ouabain in the presence of PKC inhibitors. Ouabain (30 μM) was added during exposure to the inhibitors. A. Effects of 5 μM H-7 on the contractile force and sinus rate. B. Effects of 100 nM staurosporine.

Table 2. Effects of phorbol esters on the inotropic and chronotropic effects in rat right atria in the presence of protein kinase C inhibitors.

	n	*Contractile force*	*Sinus rate*
H-7 5 μM	14	− 12·7 ± 1·5***	− 2·1 ± 1·5
+ PDB 100 nM	4	9·1 ± 9·1	10·5 ± 5·2
+ TPA 100 nM	5	7·5 ± 8·4	1·6 ± 4·4
+ PDD 100 nM	5	− 5·9 ± 4·7	− 0·1 ± 2·3
Staurosporine 100 nM	23	− 14·4 ± 3·6**	− 10·2 ± 1·7***
+ PDB 100 nM	8	− 9·9 ± 4·9	0·1 ± 2·3
+ TPA 100 nM	7	1·0 ± 2·0	− 5·8 ± 2·6
+ PDD 100 nM	8	− 10·0 ± 4·3	− 6·1 ± 2·7

Values (%) represent mean ± s.e.m. PDB: 4-β-phorbol-12,13-dibutyrate. TPA: 12-*O*-tetradecanoyl-phorbol-13-acetate. PDD: 4-α-phorbol-12,13-didecanoate. **$P < 0.01$, ***$P < 0.001$, with respect to control value.

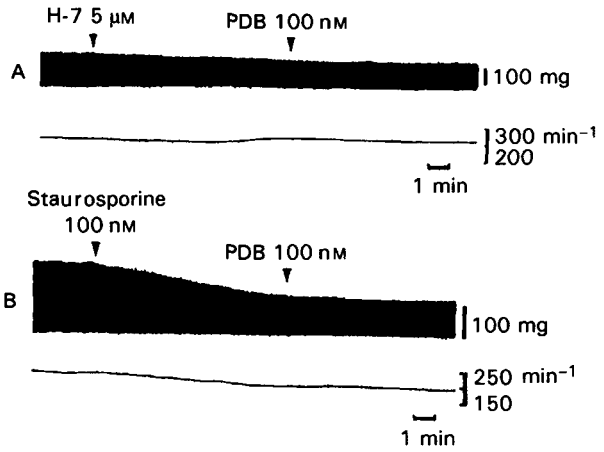

FIG. 4. Blockade of the PDB-induced effects by PKC inhibitors. A. PDB in the presence of 5 μM H-7. B. PDB in the presence of 100 nM staurosporine.

the inotropic and chronotropic effects of ouabain to any significant extent. In contrast, staurosporine (100 nM) significantly inhibited the inotropic effect by 61·9 ± 3·1% (n = 5, $P < 0.01$) as compared with that of 30 μM ouabain alone, but did not affect the sinus rate.

To confirm whether the responses to phorbol esters are due to PKC stimulation, phorbol esters were administrated in the presence of PKC inhibitors. PDB was chosed as a PKC stimulator, since PDB was found to be most potent in previous studies (Satoh & Hashimoto 1988; Satoh 1992, 1994b, 1995; Satoh et al 1992). Both H-7 (5 μM) and staurosporine (100 nM) blocked the PDB-induced responses (Figs 4A and B). The average values (including the effects of other phorbol esters) are summarized in Table 2. Also, addition of PKC inhibitors to phorbol esters was carried out. Staurosporine (100 nM) significantly decreased the force in the presence of PDB and TPA (Table 1).

Discussion

The modulation of the inotropic and chronotropic effects of ouabain and PKC stimulation was examined in rat isolated right atrial muscles. The following observations were made: ouabain caused a positive inotropic effect, but had less or no effect on the sinus rate; ouabain-induced effects were reduced by staurosporine, but not by H-7 of the PKC inhibitors; PDB increased the ouabain-evoked positive inotropic and chronotropic effects; phorbol esters (PDB and TPA) alone caused positive inotropic and chronotropic effects, and PDD (an inactive analogue of phorbol esters) had no effect; PKC inhibitors blocked the effects induced by phorbol esters.

Ouabain is a well-known cardiotonic drug, due to a specific Na^+/K^+ ATPase inhibition associated with an Na^+ pump (Lee & Fozzard 1975; Akera & Brody 1978). As a result, Na^+ increases close to the inner side of the plasma membrane, and leads to a decrease in Ca^{2+} efflux through the Na^+/Ca^{2+} exchange (Langer et al 1979; Isenberg & Klockner 1980). However, the therapeutic levels (relatively low concentrations) of glycosides stimulate the pump activity (Peters et al 1974). Cassaic acid mustard (CAM), an inhibitor of Na^+/K^+ ATPase, does not necessarily produce a positive inotropy (Rhee et al

1976). Recently, it has been reported that Na^+ pump current can be separated into ouabain-sensitive and -insensitive components (Ishizuka et al 1996). The properties of high and low affinity components of Na^+ pump current are consistent with the presence of different Na^+/K^+ ATPase isoforms. In addition, an enhancement of I_{Ca} by digitalis has been reported (Lederer & Eisner 1982; Marban & Tsien 1982), but ouabain inhibits I_{Ca} and I_K in cardiac myocytes (Josephson & Sperelakis 1977; Satoh 1994a). Thus, if ouabain causes no enhancement of I_{Ca} and minor inhibition of Na^+/K^+ ATPase, the positive inotropic effect must be associated with other mechanisms. Digitalis indirectly causes the negative chronotropic and dromotropic effects in-situ, due to the stimulation of parasympathetic nerves. In the present experiments using isolated preparations, thus, ouabain may have less or no effect on the sinus rate.

PKC stimulation by phorbol esters also inhibits I_{Ca} and I_K in several cardiac myocytes (Satoh 1992, 1994b, 1995), and causes a negative inotropic effect (Satoh et al 1992). The negative effect might be consistent with the report that PKC decreases actin-myosin interaction through an alteration of myofilament proteins (Lester et al 1996). In vascular smooth muscle cells, it enhances I_{Ca} (Fish et al 1988; Mironneau et al 1991). Satoh & Sperelakis (1995) showed that phorbol esters also inhibited I_{Ca} in cultured aortic A7r5 cells. The phorbol esters increase the contraction of vascular smooth muscles, which can contract even in the absence of extracellular Ca^{2+} (pharmacomechanical coupling). It has recently been reported that PKC stimulation leads to high sensitivity of the contractile proteins to Ca^{2+} in vascular smooth muscles (Jiang & Morgan 1987; Nishimura et al 1990). The relation between pCa and contraction force is shifted to the left in the presence of PDB. Thus, the actions induced by phorbol esters are conflicting among the different tissues and even in the same cells. In the present experiments using rat atrial muscles PKC stimulation had a positive inotropic effect.

Ouabain inhibits Na^+/K^+ ATPase, but PKC phosphorylates the α-subunit of the Na^+/K^+ pump and elevates the activity (Bertorello et al 1991). Although the enhancement of pump activity may not increase the force (Noble 1980; Vassalle 1986), both ouabain and phorbol esters in this study had a positive inotropic effect. The PKC stimulation by phorbol esters increased the positive inotropic effect evoked by ouabain. PKC inhibitors blocked the effects induced by phorbol esters, and decreased the positive inotropic effect induced by ouabain.

PKC inhibitors are not specific, but are sensitive to PKA and PKG as well as PKC (Garland et al 1987). Phosphopeptide mapping and autoradiography have recently revealed that PKA and PKC phosphorylate the same site on troponin C-protein to a similar extent (Venema & Kuo 1993). These inhibitors would depress the activities, and decreased the contraction in the present experiments, consistent with the results in rabbit SA nodal cell (Satoh 1994b, 1995; Satoh & Hashimoto 1988), guinea-pig ventricular cells (Satoh 1992), and canine ventricular muscles (Satoh et al 1992). The K_i value of H-7 was 3-6 μM for cyclic nucleotides such as PKA, PKG and PKC. H-7 has less activity as an inhibitor of myosin light chain kinase ($K_i = 97$ μM). Staurosporine, a microbial product, has been shown to exert a potent and relatively specific inhibitory effect on PKC, as compared with H-7 (Tamaoki et al 1986). In the present experiments, addition of PDB to ouabain enhanced the contraction and increased the resting tension. TPA had the same tendency, although not to a significant extent. The more potent actions of PDB than TPA were also consistent with our previous observations (Satoh & Hashimoto 1988; Satoh & Sperelakis 1991, 1995; Satoh 1992, 1994b, 1995; Satoh et al 1992). PDD, an inactive PKC stimulator, did not affect contractile force or resting tension. PKC inhibitors blocked the phorbol esters-evoked responses, and staurosporine inhibited the ouabain-induced positive inotropic effect (by about 27%). Therefore, these results indicate that not only phorbol esters but also ouabain can activate PKC which would result in the production of cardiotonic actions. We conclude that the positive inotropic effect induced by ouabain may be associated (but only in part) with PKC stimulation and that this effect might not necessarily result from the modulation of Na^+/K^+ pump activity.

In the presence of both PDB and ouabain, the resting tension was gradually elevated, consistent with results in canine Purkinje fibers (Satoh 1992). These findings indicate that addition of phorbol esters would increase the $[Ca]_i$ level (although the possibility of altered Ca^{2+}-sensitivity may exist), and then cause cardiotoxicity. Actually, Satoh (1994b) showed that phorbol esters elevated $[Ca]_i$ in rabbit SA nodal cells, using a Ca^{2+}-sensitive fluorescent dye, fura-2. The evidence is supported by a decrease in only the fast component of the time constants of inactivation phase for I_{Ca} in SA nodal cells (Satoh 1994b), in young embryonic chick ventricular cells (Satoh 1995) and also in rat aortic vascular smooth muscle cells (Satoh & Sperelakis 1991). The fast component is highly sensitive to the change in $[Ca]_i$ level (Eckert & Chad 1984). In addition, phorbol esters elicited arrhythmias in rabbit SA nodal cells (Satoh & Hashimoto 1988; Satoh 1994b). Since the PKC stimulation has been reported to regulate Na^+/H^+ exchange (which results in enhancement of Na^+/K^+ pump activity), it might lead to elevation of $[Ca]_i$ level via Na^+/Ca^{2+} exchange.

Recently many PKC isoenzymes have been identified. They are divided into two subgroups: Ca^{2+}-dependent classical PKC; and Ca^{2+}-independent neo PKC (Hug & Sarre 1993). Biochemical characterization of these PKC isoenzymes could be one prerequisite for the elucidation of their distinct roles of the still unclear mechanisms. Also, digitalis exhibits other additional actions. It causes a transient increase of $[Ca]_i$ that precedes tension development (Allen & Blinks 1978). Ouabain also increases atrial natriuretic peptide (ANP) secretion, which is a potent hypotensive hormone (Schiebinger & Cragoe 1993). ANP might result in the I_{Ca} inhibition. Thus, further experiments are required to elucidate the detailed mechanisms of digitalis.

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