Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 72-78

INVOLVEMENT OF PHOSPHOINOSITIDE TURNOVER IN OUABAIN INOTROPISM

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Received May 25, 1993

SUMMARY : We examined whether ouabain activates phopholipases and reinforces contraction force of papillary muscles through resultant second messengers. 2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), the inhibitor of phospholipase C (PLC), abolished the ouabain inotropy in rabbit papillary muscles. Calphostin C, the specific inhibitor of protein kinase C (PKC), also depressed the ouabain inotropy. 12-O-Tetradecanoylphorbor-13-acetate (TPA), the specific activator of PKC, enhanced the beat-to-beat phasic contractility at low concentrations. Radioenzymatic assay revealed that ouabain treatment doubled diacylglycerol (DG) content in excised papillary muscles. We concluded that ouabain activates PLC, and the resultant second messenger, DG, augments the cardiac contraction force through activation of PKC.

Digitalis and other cardiac glycosides augment cardiac contraction force (ouabain inotropism), and they have been unrivalled in value for the treatment of heart failure. Their mechanisim, however, is still controversial (1). They are specific inhibitors of Na^+ pump and raise the intracellular Na^+ concentration, resulting in the increase in the intracellular Ca^{2+} concentration ($[Ca]_i$) through Na^+-Ca^{2+} exchange. The increased force is usually attributed to this rise in $[Ca]_i$. However, ouabain activates phospholipases in various tissues such as taenia coli (2, 3), cardiac muslces (4, 5), and hypothalamus (6). Resultant prostaglandins are essential mediators for ouabain-induced contraction in taenia coli (2, 3). In rat

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Abbreviations: DG, diacylglycerol; H-7, 1-(5-Isoquinolinylsulfonyl)-2-methylpiperizine; IP_3, inositol trisphosphate; NCDC, 2-Nitro-4-carboxyphenyl -N,N-diphenylcarbamate; PI, phosphatidyl-inositol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; TPA, 12-O-Tetradecanoylphorbor-13-acetate.

0006-291X/93 $4.00

papillary muscles, on the other hand, ouabain can activate PLC through Ca^{2+} influx and releases IP_3, resulting in the putative enhancement of cardiac force (5). The present paper directly indicates the involvement of a PLC-DG pathway in the ouabain inotropy.

MATERIALS AND METHODS

Perfused Papillary Muscles.

Rabbits were anaesthetized with 25% urethane, and the ventricular septum with papillary muscles attached was excised. This massive preparation was perfused through the septal artery by a modified Tyrode solution mixed with 1% blood at room temperature, 25°C. The composition of the modified Tyrode solution was as follows:Na^+ 149 (mM), K^+ 2.7, Ca^{2+} 1.8, Mg^{2+} 1.1, Cl^- 143, HCO_3^- 12, PO_4^{2-} 0.4, glucose 28. The Tyrode perfusate was equilibrated with a mixture of 95% O_2-5% CO_2. One of the papillary muscles was connected to a strain gauge transducer (Shinko, UL-10) and was subjected to a constant resting tension of 5 mN. The muscle was driven at 0.5 or 1 Hz for at least 1 hour to allow stabilization before the experimental procedures were begun. The drugs were dissolved in the perfusate. In order to avoid indirect effects of endogenous substances released, the following antagonists were mixed in the Tyrode solution: phentolamine (α -adrenergic antagonist, 1 μ M. Ciba-Geigy), propranolol (β -adrenergic antagonist, 1 μ M. Sigma), cimetidine (the antagonist against histamine H_2 receptor, 20 μ M. Tagamet from Fujisawa Pharmaceuticals), metoclopramide (dopamine receptor antagonist, 50 μ M. Prinperane from Fujisawa Pharmaceuticals). These antagonists slightly depressed the control contraction, but influenced neither the ouabain inotropy nor actions of inhibitors used.

The present perfused papillary muscles develop much stronger twitch tensions for a longer period than conventional strips of excised papillary muscles (7). 1% blood and the glucose concentration which is 10 times that of the normal Tyrode also reinforced the contractility and made a long experiment (as long as 7 hours) possible.

Excised Papillary Muscles.

We measured the change of DG content due to ouabain in papillary muscles, and obtained its time-course. The perfusion preparation used for force experiments above was not adequate for such biochemical analyses because of 2 reasons: (1) its massive preparation makes it difficult to stop biochemical actions of ouabain by rapid freezing, and (2) dissection itself stimulates PLC. We, therefore, prepared small excised papillary muslces from guinea pigs and started experiments after 2 hrs when PLC activity returned to normal. Small thin samples from guinea pigs were more desirable for biochemical experiments than excised samples from rabbits because thick samples from rabbits in a superfusion condition are accompanied by O_2 deprivation which activates PLC unphysiologically.

The ouabain action was stopped after a certain period of time by rapidly immersing the excised sample in cold methanol with dry ice. The assay of DG was done by Amersham's *sn* -1,2-diacylglycerol assay reagent system, RPN 200. Radioenzymatic assay was done using diacylglycerol kinase which converts DG to [^{32}P] phosphatidic acid in the presence of [^{32}P] - γ -ATP. [^{32}P] phosphatidic acid was separated by using thin-layer chromatography and was quantitated by liquid scintillation spectrometry.

In order to make a consistent discussion using the same type of preparation from the same species, we performed all of the force experiments using these excised samples;we confirmed that PLC activation is involved in ouabain inotropy in such conventional preparations as well as in perfused ones, although the excised preparations were less stable than the perfused ones.

Miscellaneous.

NCDC (Sigma, St. Louis, USA), Calphostin C (a gift from Dr. T. Tamaoki of Kyowa Hakko, Tokyo, Japan), ouabain (Nakarai Chemicals, Kyoto, Japan) were

dissolved in perfusates. TPA (Funakoshi Chemicals, Tokyo, Japan) was kept as a 10 mM stock solution in dimethylsulfoxide. Dimethylsulfoxide itself did not show any significant inotropic action on the present tissue at the concentrations used. H-7 (Seikagaku Kogyo, Tokyo, Japan). Sphingosine and dioctanoylglycerol (Sedary Res. Lab., Ontario, Canada).

RESULTS

Ouabain Inotropy through Activation of Phospholipase C.

NCDC is an inhibitor of PLC. It has been used in various tissues to examine the involvement of PLC in autacoid actions (8, 9), including positive inotropism by antigens (10) and interleukin-2 (11) in cardiac muscles. We used NCDC in order to examine whether PLC is involved in ouabain inotropism. Pretreatment of the arterial perfusion preparations of rabbit papillary muscles with 10 μ M NCDC completely abolished the positive inotropy of 2 μ M ouabain (Figure 1). The effect of NCDC was reversible. In all five experiments performed, NCDC always blocked ouabain inotropism. NCDC itself augmented the control contraction force slightly (less than 10%). We measured increase in the contraction height by 10 min application of ouabain and found that the positive inotropism is always blocked by NCDC; 125 ± 11% (mean ± SEM, n=5) in control to 82 ± 17% (n=5) in the presence of 10 μ M NCDC, where 100% is the contraction height before ouabain application. NCDC not only blocked the positive inotropy, but reversed the ouabain inotropy to a negative direction (Figure 1, lower trace). This reflects the action of prostanoids produced with ouabain, a point which we will discuss later. NCDC of 1 μ M suppressed ouabain inotropism by 70%. The inotropy by ouabain of one order lower concentration was also blocked by NCDC.

PLC produces a second messenger, DG. DG activates PKC. The effects of some PKC inhibitors on ouabain inotropism, therefore, were examined. Sphingosine

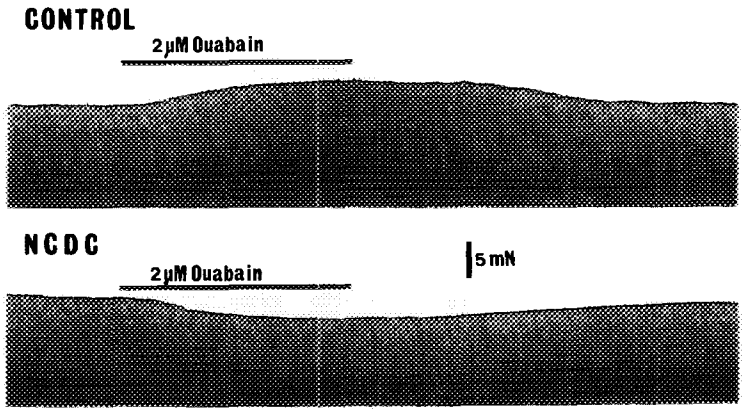

Figure 1. NCDC blocks a positive inotropic effect of ouabain in a rabbit papillary muscle. Ouabain: 2 μ M, 7 min. The stimulation freguency was 0.5 Hz. The tissue was treated with 10 μ M of NCDC for 30 minutes prior to challenge with ouabain. The ouabain inotropy recovered after removing NCDC.

and H-7, both at 10 μ M, suppressed the inotropism of 2 μ M ouabain by approximately 70% in excised papillary muscles. 2 μ M of ouabain, however, is 2 orders higher than the clinical dosage for the treatment of heart failure. There is a possibility, therefore, that only such high conentration of ouabain could activate PLC. Figure 2 shows Calphostin C, a new specific inhibitor of PKC, has an inhibitory influence on a clinical low dosage of ouabain, 20 nM;Calphostin C can inhibit PKC without influencing A-Kinase or calmodulin-kinase and is one of the most useful PKC-specific inhibitors (12-15). Such a low concentration of ouabain still caused a weak but reproducible inotropism. The contraction heights before and after 20 min of ouabain application were 117% in Control (upper trace), and 104% in the Calphostin C-treated (lower trace), respectively. The similar results were obtained in 4 more experiments, and Calphostin C significantly suppressed the ouabain inotropy; 114 ± 4% (mean ± SEM, n=5) in control to 101 ± 3% (n=5) in the presence of 100 nM Calphostin C, where 100% is the contraction height before ouabain application. Calphostin C blocked the inotropism of 2 μ M ouabain also.

When TPA, a specific activator of PKC, was applied at concentrations of 10-1000 nM to the papillary muscles, the contraction force was enhanced reproducibly (Figure 3). The degree of the enhancement was comparable to that produced by 2 μ M ouabain. The dose-dependence of the TPA action, however, was not clear. In agreement with Teutch et al. (16), 1,2-dioctanoylglycerol, a kind of synthetic diacylglycerol, also enhanced the contraction force by approximately 10% at 10 μ M.

Ouabain Raises Diacylglycerol Content.

We measured the change of DG content due to ouabain in excised papillary muscles from guinea pigs. Figure 4 indicates that 2 μ M ouabain rapidly increases

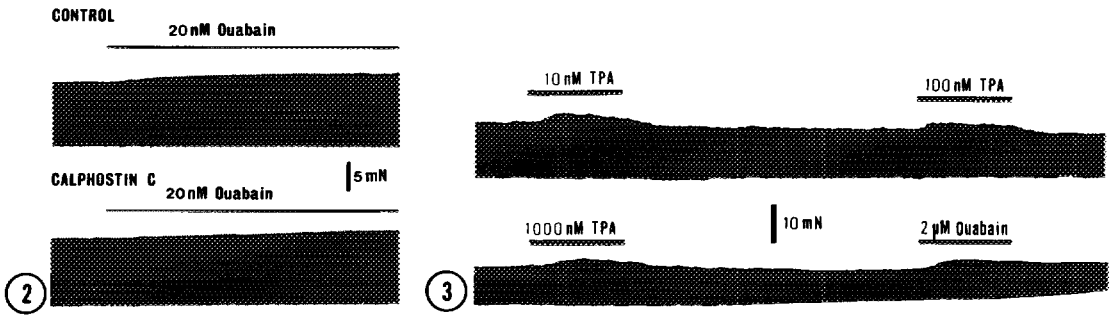

Figure 2. Suppression of the ouabain inotropy with Calphostin C, a PKC inhibitor. The perfused papillary muscle was driven at 1 Hz. The preparation was pretreated with Calphostin C of 0.1 μ M for 30 min, and then was applied with 20 nM ouabain. The effect of Calphostin C was reversible. The horizontal bars in the figure indicate ouabain application and the time period of 20 min.

Figure 3. TPA produces positive inotropism in the perfused preparation, comparable to the ouabain effect. The drugs were applied for 10 min with intervals of 30 min. An unphysiological contracture appeared in the ouabain experiment. Stimulation protocol was the same as in Figure 1.

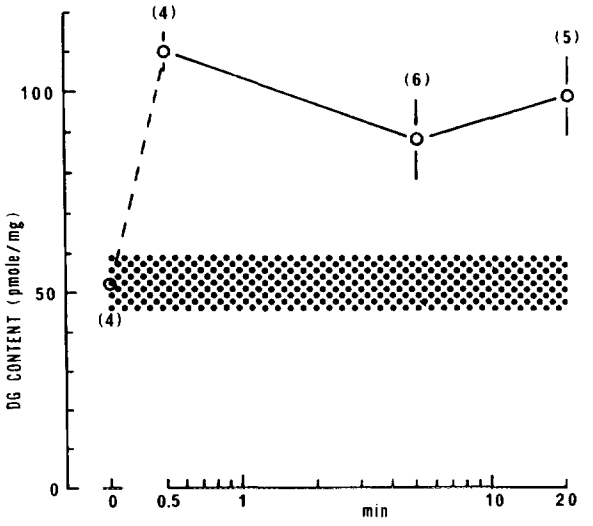

Figure 4. Stimulation of diacylglycerol (DG) production in excised papillary muscles from guinea pigs by ouabain (2 μ M). Incubation of the tissue in a ouabain medium started at 0 min. The stippled area represents the control content of DG ± SEM in the absence of ouabain. The data are displayed as mean ± SEM. Number of experiments in parentheses.

the DG content in the muscles. The DG level reached its maximum at around 30 sec, and this rapid rise is in agreement with other reports on PLC activation kinetics. We concluded that ouabain activates PLC and that its product, DG, increases the cardiac contractility through the activation of PKC.

DISCUSSION

A high concentration (2 μ M) of ouabain was used in most of the present experiments in order to obtain clear-cut positive inotropism in a short period and to avoid spontaneous deterioration of papillary muscles. We confirmed, however, that the present conclusions could be obtained for a lower dosage of ouabain in some inhibitor experiments; the PKC inhibitor depressed a positive inotropism by ouabain of the clinical low dosage (Figure 2). The high concentration of ouabain, therefore, seems unlikely to be the cause of the PLC activation.

α_1-adrenergic receptor agonists elicit a positive inotropic effect in association with acceleration of PI hydrolysis. In fact, phenylephrine, a specific α_1-adrenergic agonist, significantly increased the DG content in the guinea pig papillary muscles, and NCDC blocked its inotropism. On the other hand, β_1-adrenergic inotropism was not influenced by NCDC, Calphostin C, H-7, nor by sphingosine. NCDC is not always a specific PLC inhibitor and can block other esterases, but these results taken together suggest that NCDC blocked PLC in papillary muslces. All of other evidences shown here also support the scheme that ouabain activates PLC.

We applied a K^+-free medium, another procedure to suppress a Na^+ pump, resulting in a profound positive inotropism. This inotropism was suppressed to 50% by H-7 of 10 μ M reversibly. The nature of the coupling between the Na^+ pump inhibition and PLC activation, however, is not understood. Two hypotheses have been proposed concerning this coupling mechanism. Otani et al. (6) claimed that the Na^+ pump inhibition activates PLC through the increase in $[Ca]_i$ because PLC is a Ca^{2+}-dependent enzyme. The other hypothesis is that there is an interaction at the level of the plasma membrane where Na^+, K^+-ATPase inhibition activates a PI specific lipase: Coburn (2) claimed this theory in the ouabain contraction of taenia coli.

The effect of PKC activators on cardiac force has been controversial. Teutsch et al. (16) reported that a synthetic DG (1, 2-dioctanoyl-glycerol) produces a positive inotropic response in guinea pig atria. On the contrary, Leatherman et al. (17) and Dosemeci et al. (18) found that TPA had a negative inotropic effect. We too observed such a negative inotropy frequently when we applied high concentrations of TPA to conventional excised preparations. However, positive inotropy was always obtained when TPA was applied at low concentrations (1-1000 nM) to the perfusion preparation (Figure 3). A PLC-PKC pathway is regulated by a negative feed-back mechanism. One explanation for the discrepancy, therefore, is that the negative inotropism of TPA of high concentrations in conventional preparations is caused by an unphysiological feed-back mechanism.

Ouabain enhances the opening of voltage dependent Ca^{2+}-channels (19, 20) without changing the calcium sensitivity of regulatory contractile proteins, or sarcoplasmic reticulum Ca^{2+} uptake. Marban and Tsien (21) reported that a small rise in the diastolic $[Ca]_i$ due to ouabain increases the Ca^{2+} current. They postulated two candidates which link the small rise in $[Ca]_i$ to the Ca^{2+} current. One is Ca^{2+}-calmodulin and the other is a membrane phospholipid metabolite. Our results favor the latter scheme: DG, one of the phospholipid metabolites, causes the ouabain inotropism. The effect of DG and other PKC activators on Ca^{2+}-channels, however, is again controversial: augmentation (18), no effect (22), and augmentation followed by suppression (23, 24). The scheme of Ca^{2+} channel opening by an ouabain-DG pathway, therefore, remains to be settled.

Moffat et al. (3) indicated that acetylstrophantidin, one of cardiac glycosides, activates PLA_2, and resultant prostaglandins may play a role in ventricular arrhythmia. We have recently analysed the effects of some PLA_2- and prostanoid-related reagents on ouabain inotropy. We reached a conclusion that ouabain activates PLA_2 as well as PLC, and resultant prostanoids cause rather a negative inotropism (in preparation). Therefore, we suppose that the negative inotropism of ouabain in the presence of NCDC (Figure 1, lower trace) probably reflects the suppressive effect due to prostanoids, unmasked by the inhibition of PLC.

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