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PHORBOL 12,13-DIBUTYRATE PRODUCES NEGATIVE INOTROPISM AND SELECTIVE ANTAGONISM OF RESPONSES TO ADRENOCEPTOR AGONISTS IN RAT ATRIA

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Abstract—Rat atria loaded *in vitro* with the dye INDO-1 produced fluorescence signals indicative of changes in cytoplasmic calcium ion concentation ($[Ca^{2+}]_c$). Such atria showed systolic/diastolic fluctuations indicative of a systolic rise and a diastolic fall in both tension and $[Ca^{2+}]_c$. Positively inotropic responses of the atria to isoprenaline, phenylephrine, ouabain or 4-aminopyridine were associated with fluorescence changes indicative of increased systolic increments in $[Ca^{2+}]_c$. Treatment of atria with phorbol dibutyrate, on the other hand, produced negative inotropism and fluorescence changes indicative of declining systolic increments in $[Ca^{2+}]_c$. Pretreating atria with phorbol dibutyrate diminished both the inotropic and fluorescence responses to subsequent treatment with phenylephrine or isoprenaline, although responses to ouabain or 4-aminopyridine were unchanged. Exposure of atria to a selective inhibitor of protein kinase C mitigated the effects of pretreatment with phorbol dibutyrate, but failed to modify responses to phenylephrine or isoprenaline that were produced in the absence of phorbol dibutyrate.

Treatment of several mammalian tissues, including the myocardium, with α-adrenoceptor agonists causes a redistribution and activation of a family of calcium- and phospholipid-dependent enzymes known as protein kinase C (PKC†) [1–3]. The possibility that activation of PKC might mediate the positively inotropic responses shown by many types of cardiac muscle to α-adrenoceptor agonists via the intracellular production of diacylglycerol has been proposed [4, 5], but remains uncertain [6, 7]. Although treatment of the myocardium *in vitro* with various PKC-activating phorbol esters sometimes causes a positively inotropic response [7], more usually a paradoxical and negatively inotropic response is seen [3, 8–12]. Some workers, however, have found no inotropic effect with these phorbol esters [6, 13]. Whether, or to what extent, these varying inotropic responses to phorbol esters depend upon a redistribution and activation of PKC in these hearts is unclear [11]. Unfortunately, most of the available inhibitors of PKC are markedly deficient in specificity [14, 15]. The recent discovery of a highly selective inhibitor of PKC [14], in the form of 3-(*N*-3′-*n*-propylthiomethanimidaminoindolyl)-4 - (*N* - methylindoly) - pyrrolidin - 2,5 - dione (Ro31,8220), provided an opportunity to re-examine the possible involvement of PKC as a mediator of inotropic responses to a variety of chemical agents.

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† Abbreviations: $[Ca^{2+}]_c$, cytoplasmic calcium ion concentration; PKC, protein kinase C; Ro31,8220, 3-(*N*-3′-*n*-propyl thiomethanimidamino indolyl)-4-(*N*-methyl-indolyl)-pyrrolidine-2,5-dione; PDB, 4-β-phorbol, 12,13-dibutyrate; PDD, 4-α-phorbol 12,13-didecanoate; WB4101, 2 - (2,6 - dimethoxyphenoxyethyl)aminoethyl - 1,4 - benzodioxane) hydrochloride.

Rat atria were chosen for this study, since they display strongly positive and monophasic inotropic responses to the α-adrenoceptor agonist drug, phenylephrine [16–19]. Rat atria also permit continuous monitoring of the intracellular calcium ion concentration by a fluorimetric means [20], and this technique has been used already to explore the mechanism of action of certain inotropic drugs [21, 22].

MATERIALS AND METHODS

Atria were isolated from rat hearts and mounted on a plastic holder, as described in detail previously [20]. Briefly, the atria were bathed, unless otherwise specified, with a superfusate of the following composition (mM): NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 10, glucose 10, and gassed with 95% 0_2 plus 5% CO_2. Atria were stimulated throughout an experiment at 3 Hz with 2 msec square wave pulses, each of 10 V. A thread sutured to the left atrial appendage was connected to a force displacement transducer (type SB-1T, Nihon Kohden) at a diastolic tension of 100 mg. Tension was recorded via a DC amplifier (type 5242) coupled to a heated stylus recorder (MultiTrace 2) made by Lectromed. Atria were allowed to equilibrate for 1 hr before dye loading or alteration to superfusate composition.

Dye loading and fluorescence measurement. Atria were loaded with the acetoxymethyl ester of INDO-1 (Sigma) as described previously [20]. Dye-loaded atria, on the plastic holder, were positioned diagonally across a 1 cm section quartz cuvette and kept at 34° in a specially designed fluorimeter with one excitation monochromator and two emission monochromators, each with a photon-counting

multiplier tube (the Alphascan system, Photon Technology International). Epifluorescence emissions were collected digitally at 60 data points/sec from the front space and one side face of the cuvette. Excitation was at 360 nm, and the two emission monochromators were set at 400 and 500 nm. The intensity of fluorescence (F) emitted at 400 nm is increased by a rise in calcium ion concentration, but diminished at 500 nm. Changes in the ratio of emission intensities of these two wavelengths ($F400/500$) are a measure of changes in cytoplasmic calcium ion concentrations [$(Ca^{2+})_c$] but are not convertible to absolute concentration values [23]. Before dye loading, however, the combined tissue and instrumentation autofluorescence was recorded and filed in computer memory, to be deducted later from signals generated by dye-loaded atria. All values in this report have been corrected in this way. Tissue autofluorescence needed to be recorded for each experimental intervention, however, as it varied in different superfusates. There was a systolic rise and a diastolic fall in the corrected $F400/500$ ratio of dye-loaded atria with each applied electrical pacing stimulus, as described previously [20].

Calculation of the magnitude of inotropic and fluorescence response. In the present report both the atrial systolic developed tension and the $F400/500$ ratio changes produced in response to each of the experimental interventions were expressed as percentages, calculated with respect to corresponding values displayed by the atria prior to the relevant intervention. Each positively inotropic agent studied gave a substantially constant percentage value in successive responses elicited at 50 min intervals for up to 10 hr. Absolute values for these same parameters were less consistent. Mean values for responses of different groups of atria were considered to be significantly different from each other in Students t-test if the null hypothesis was rejected because $P < 0.50$.

Chemicals. Unless otherwise stated below, chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.) and dissolved in water for use. L-Phenylephrine and DL-propranolol were obtained as hydrochlorides, and ouabain as the octahydrate. 4-Aminopyridine and 3,4-diaminopyridine were dissolved in sufficient hydrochloric acid to give a neutral solution. 4-β-Phorbol 12,13-dibutyrate (PDB) and 4-α-phorbol 12,13-didecanoate (PDD) were dissolved in dimethyl sulphoxide and kept in the dark until used. Racemic isoprenaline hemisulphate was a gift from Abbott Laboratories (Queenborough, U.K.), and was dissolved in water containing ascorbate to avoid oxidation. Prazosin hydrochloride was a gift from Pfizer Ltd (Sandwich, Kent). 2-(2,6-Dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) hydrochloride (WB4101) and chloroethylclonidine hydrochloride were purchased from Research Biochemicals Inc. Ro31,8220 was a gift from Drs J. Waterfall and G. Lawton of Roche Products Ltd (Welwyn Garden City, U.K.), and was dissolved in dimethyl sulphoxide for use.

RESULTS

Effects of phenylephrine

Exposing atria to phenylephrine (10–50 μM) caused a rapidly developing and concentration-dependent positively inotropic response. This was associated with an equally rapid increase in systolic increments in the $F400/500$ ratio. These effects of phenylephrine disappeared rapidly after returning atria to drug-free superfusate. Repeated short periods of exposure of the atria to 50 μM phenylephrine at 50 min intervals for up to 10 hr yielded a series of fairly constant tension and fluorescence responses.

Responses to phenylephrine were not significantly altered by pretreating the atria for 10 min with 5 μM propranolol. In view of this, 5 μM propranolol was routinely added to the superfusate when phenylephrine was used in the remaining experiments. Both the tension and the fluorescence changes produced in response to phenylephrine were abolished by pretreating the atria for 10 min with 0.1 μM prazosin or 0.1 μM WB4101 (Table 1). Pretreating the atria for 30 min with 10 μM chloroethylclonidine, on the other hand, failed to alter tension or fluorescence responses to 50 μM phenylephrine to a significant extent.

Effects of isoprenaline

Exposing atria to isoprenaline (0.2–1.0 μM) caused a rapidly developing and concentration-dependent positively inotropic response that was associated with an equally rapid increase in systolic increments in the $F400/500$ ratio, confirming previous findings with this preparation [20, 24]. These effects of isoprenaline disappeared rapidly after returning atria to a drug-free superfusate. Repeated short periods of exposure of the atria to 1 μM isoprenaline at 50 min intervals for up to 10 hr yielded a series of fairly constant tension and fluorescence responses. Although 1 μM isoprenaline caused a positively inotropic response of similar magnitude to that caused by 50 μM phenylephrine (208% with isoprenaline vs 193% with phenylephrine), systolic increments in the $F400/500$ ratio were increased to quite different extents (235% with isoprenaline vs 162% with phenylephrine).

Atrial responses to isoprenaline were abolished by pretreatment for 10 min with 5 μM propranolol (Table 1). Responses to 1 μM isoprenaline were not altered significantly, however, by pretreatment for 10 min with either 0.1 μM prazosin or 0.1 μM WB4101. In view of this, 0.1 μM prazosin was routinely added to the superfusate in the remaining experiments with isoprenaline.

Effects of phorbol esters

Exposing atria to 0.1 μM PDB caused a slowly developing and negatively inotropic response that was maximal after about 20 min (Fig. 1). Returning atria to drug-free superfusate at this stage permitted systolic developed tension values to return slowly towards their pre-PDB value over the next 2 hr (Fig. 1). Exposing atria to PDB at the higher concentration of 1 μM caused a negatively inotropic response of similar magnitude to the lower concentration (77% at 1 μM vs 79% at 0.1 μM, $P > 0.05$), although responses at the higher concentration persisted for even longer after removal of PDB from the superfusate. During the first 30 min of exposure to 0.1 μM

Table 1. Effects of adrenoceptor agonists on the atria

Agonist		Antagonist (μM)			Systolic	
Name	μM	Prazosin	WB4101	Propranolol	Developed tension (% control)	Increment in F400/500 ratio (% control)
Phenylephrine	10	0	0	0	157 ± 16	138 ± 8
Phenylephrine	10	0.1	0	0	102 ± 10	98 ± 6
Phenylephrine	10	0	0.1	0	100 ± 7	101 ± 9
Phenylephrine	50	0	0	0	191 ± 18	161 ± 12
Phenylephrine	50	0.1	0	0	109 ± 8	100 ± 6
Phenylephrine	50	0	0.1	0	101 ± 9	98 ± 7
Isoprenaline	0.2	0	0	0	166 ± 15	180 ± 16
Isoprenaline	0.2	0	0	5	100 ± 8	101 ± 9
Isoprenaline	1	0	0	0	210 ± 19	231 ± 20
Isoprenaline	1	0	0	5	106 ± 9	100 ± 10

Control values (100%) were obtained immediately prior to the 5 min period of exposure to the adrenoceptor agonists. Antagonists were present in the superfusate where indicated for 10 min immediately prior to and during exposure to the adrenoceptor agonists.

Tabulated values are the means of eight observations ± SEM. Pairs of corresponding values that are significantly different ($P < 0.05$) are bracketed together.

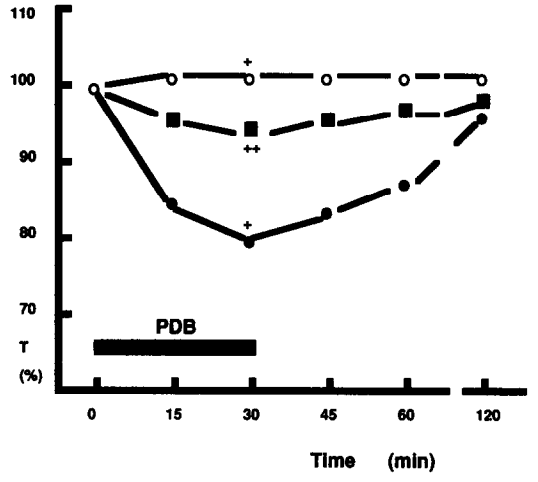

Fig. 1. Atrial systolic developed tension (T) responses during (horizontal bar) and after 30 min of contact with 0.1 μM PDB (●). A control group (○) was exposed throughout to PDB-free superfusate. Another group (■) was treated with 1 μM Ro31,8220 for 30 min prior to, as well as during and after, the PDB treatment. Values marked + are significantly different from each other ($P < 0.05$). The value marked ++ was significantly different from the 30 min value for PDB-treated atria that had not been exposed to Ro31,8220 (●). There were eight or 10 observations at each data point.

PDB systolic increments in the $F400/500$ ratio declined only slighly (to 88% of control, $P > 0.05$). A higher concentration of PDB (1 μM), however, significantly reduced systolic increments in the $F400/500$ ratio (to 66% of control, $P < 0.05$), while at the same time reducing developed systolic tension significantly (to 70% of control, $P < 0.05$). Exposing atria for 30 min to PDB at 10 nM, however, caused no detectable effects.

Pretreating atria with 0.1 μM PDB for 30 min greatly reduced the previously described positively inotropic response to 50 μM phenylephrine, as well as the accompanying increase in systolic increments in the $F400/500$ ratio (Table 2). Responses to 1 μM isoprenaline were less susceptible to inhibition by PDB than inotropic responses of approximately equal magnitude to phenylephrine. Pretreating atria with PDB (1 μM for 30 min) almost abolished both the inotropic and fluorescence responses to phenylephrine, whereas the corresponding responses to isoprenaline were still substantial (Table 2). Pretreating atria for 30 min with PDB at 10 nM still reduced the inotropic response to 50 μM phenylephrine significantly (193% without PDB vs 125% with PDB, $P < 0.05$), whereas the inotropic response to 1 μM isoprenaline was not significantly affected (208% without PDB vs 197% with PDB, $P < 0.05$).

Exposing atria for 30 min to 1 μM PDD, a phorbol ester that reputedly does not activate PKC [25], failed to exert an inotropic effect and failed to alter systolic increments in the $F400/500$ ratio significantly. Moreover, atria that had been pretreated with PDD (1 μM for 30 min) showed inotropic and fluorescence responses to 50 μM phenylephrine or 1 μM isoprenaline that were not significantly different from those seen in the absence of a phorbol ester.

Effects of aminopyridines

Exposing atria to either 4-aminopyridine (0.1–1.0 mM) or 3,4-diaminopyridine (10–100 μM) rapidly elicited concentration-dependent and positively inotropic responses that were accompanied by similar increases in systolic increments in the $F400/500$ ratio. Responses to 4-aminopyridine are shown in Fig. 2, and the responses to 3,4-diaminopyridine were similar. Responses to both agents were well

Table 2. Effect of PDB on atrial responses to adrenoceptor agonists

Agonist	PDB (μM)	Ro (μM)	4AP (mM)	Systolic: Developed tension (% control)	Systolic: Increment in F400/500 ratio (% control)
Phenylephrine	0	0	0	193 ± 9*	182 ± 13*
Phenylephrine	0.1	0	0	111 ± 2†	129 ± 7†
Phenylephrine	1.0	0	0	104 ± 2†	103 ± 2†
Phenylephrine	0.1	1.0	0	190 ± 13	174 ± 14
Phenylephrine	0	1.0	1.0	158 ± 8†	150 ± 4†
Phenylephrine	0	0	1.0	160 ± 6†	150 ± 9†
Isoprenaline	0	0	0	208 ± 18‡	236 ± 19‡
Isoprenaline	0.1	0	0	140 ± 8§	160 ± 13§
Isoprenaline	1.0	0	0	137 ± 10§	153 ± 16§
Isoprenaline	0.1	1.0	0	180 ± 17	198 ± 21
Isoprenaline	0	0	1.0	184 ± 16	202 ± 23

Control values (100%) were obtained immediately prior to the 5 min period of exposure to 50 μM phenylephrine or 1 μM isoprenaline.

Tabulated values are the means of eight observations ± SEM.

A significant difference exists ($P < 0.05$) between a value marked * and corresponding values marked †, and also between a value marked ‡ and corresponding values marked §.

Atria were pretreated where indicated with PDB, Ro31,8820 (Ro) and 4-aminopyridine (4AP) for 30, 60 and 15 min, respectively, immediately prior to and during exposure to the adrenoceptor agonists.

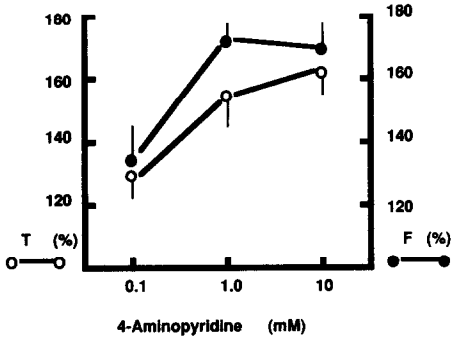

Fig. 2. Atrial systolic developed tension (T) responses (○) amd systolic increments (F) in the $F400/500$ ratio (●) produced by 4-aminopyridine. There were eight or 12 observations at each data point. Vertical bars represent SEM.

maintained in the continued presence of these drugs, but they disappeared rapidly after returning atria to drug-free superfusate. Responses of the atria to 10 mM 4-aminopyridine were insignificantly different from those elicited at 1 mM (Fig. 2). Moreover, inotropic and fluorescence responses to 4-aminopyridine at both 1 and 10 mM were smaller than those produced by 1 μM isoprenaline or 50 μM phenylephrine. Repeated exposure of atria to 1 mM 4-aminopyridine at 50 min intervals for up to 10 hr gave a series of tension and fluorescence responses that did not change significantly with time. Pretreating atria with 0.1 μM PDB for 40 min failed to alter tension and fluorescence responses to 1 mM 4-aminopyridine significantly. In this respect also, therefore, atrial responses to 4-aminopyridine contrast with those to adrenoceptor agonists, and particularly to phenylephrine.

Inotropic and fluorescence responses to 1 μM isoprenaline were insignificantly different in atria that had been pretreated for 15 min with 1 mM 4-aminopyridine and in those which had not been exposed to 4-aminopyridine. In contrast, both the inotropic and fluorescence responses to 50 μM phenylephrine were significantly smaller in 4-aminopyridine-pretreated atria than in atria that had not been pretreated in this way.

Effects of ouabain

Exposing atria to 50 μM ouabain produced a slowly developing and positively inotropic response that was maximal after 40–50 min and was associated with an approximately parallel increase in systolic increments in the $F400/500$ ratio, confirming previous findings with this preparation [22, 24]. Responses to ouabain, like those to 4-aminopyridine, but unlike those to adrenoceptor agonists, were not significantly altered by pretreating the atria for 30 min with 0.1 μM PDB.

Effects of Ro31,8220

Exposing atria to Ro31,8220 (1–10 μM) for up to 1 hr failed to produce a significant change in the magnitude of systolic developed tension elicited by each electrical pacing stimulus and failed to cause a significant change in the accompanying systolic increments in the $F400/500$ ratio. The previously described inotropic responses to 50 μM phenylephrine, 1 μM isoprenaline, 1 mM 4-aminopyridine or 50 μM ouabain likewise were not significantly changed by pretreating the atria for 1 hr with Ro31,8220 at either 1 or 10 μM. On the other hand,

Fig. 1 shows that the magnitude of the previously described inotropic response to 0.1 μM PDB was less marked in atria that had been pretreated for 1 hr with 1 μM Ro31,8220 than in atria without exposure to the PKC inhibitor (79% without vs 90% with Ro31,8220, $P < 0.05$). A 10-fold higher concentration of Ro31,8220 (10 μM for 1 hr) protected against the negatively inotropic effects of PDB no more fully than the 1 μM concentration of this PKC inhibitor.

Atria that had been pretreated with a combination of Ro31,8220 (1 μM for 1 hr) and PDB (0.1 μM for the final 30 min) gave responses to phenylephrine or to isoprenaline that were substantially larger than those elicited in the presence of PDB but without a period of pretreatment with Ro31,8220 (Table 2). Indeed, the responses to phenylephrine and isoprenaline were restored to values almost equal to those seen in the absence of PDB. Moreover, the protective effects of Ro31,8229 were selective towards PDB. This was shown by the fact that exposing atria for 1 hr to 1 μM Ro31,8220 failed to modify the ability of pretreatment with either 1 mM 4-aminopyridine or 0.1 μM prazosin to inhibit subsequent responses to 50 μM phenylephrine (Table 2).

DISCUSSION

Several reasons exist for thinking that changes which occur in *F*400/500 ratios of INDO-1 loaded atria reflect contemporary changes taking place in $[Ca^{2+}]_c$, as discussed previously [20–22]. If one accepts those reasons, the present study would suggest that the inotropic effects of phenylephrine and isoprenaline were associated with, and therefore in part at least due to, an increased systolic increment in $[Ca^{2+}]_c$. This agrees with the previous findings of several workers [26–31], although some of these investigators have noted that systolic increments in $[Ca^{2+}]_c$ were increased by phenylephrine to a smaller extent than by equally inotropic concentrations of certain other agents. Terzic *et al.* [32] found no change in systolic increments in $[Ca^{2+}]_c$ during a period of phenylephrine-induced positive inotropism. Instead, these authors attributed positive inotropism to a rise in intracellular pH. The extent to which alkalinization, or another calcium-sensitizing alteration, occurred in the present experiments is not known. The phenylephrine-induced increase in systolic increments in the *F*400/500 ratio, however, was smaller than that produced by an equally inotropic concentration of isoprenaline, suggesting either that phenylephrine had sensitized the myofibrils to calcium or that isoprenaline had exerted an opposite effect.

Two types of prazosin-blockable α-adrenoceptors have been found in the heart. At the α1A subtype WB4101 acts as a highly selective competitive antagonist, whereas at the α1B subtype chloroethylclonidine acts as a selective, albeit irreversible, antagonist [33]. Positive inotropism and the closure of potassium channels in response to phenylephrine were shown to be mediated by both α1A and α1B subtypes in ventricular myocardium from rabbits [34, 35] and rats [36, 37]. The present experiments with WB4101 and chloroethylclonidine suggest that in rat atria, on the other hand, responses to phenylephrine were mediated only by the α1A type of receptor.

That an increased systolic increment in $[Ca^{2+}]_c$ is responsible for positively inotropic responses to ouabain is now well established [22]. The present fluorescence findings with a pair of aminopyridine compounds, on the other hand, provide the first evidence that aminopyridines also increase systolic increments in $[Ca^{2+}]_c$. Aminopyridines are known to block potassium channels, and thereby to prolong the cardiac action potential [38]. In this way, aminopyridines probably increase the influx of calcium across the plasmalemma during systole, and hence increase the store of releasable calcium in the sarcoplasmic reticulum.

The PDB-induced negative inotropism seen in the present experiments confirms previous findings (see above), and was associated here with a modest reduction in systolic increments in the *F*400/500 ratio. Some workers, using isolated cardiac myocytes, have reported previously a rather larger phorbol ester-induced reduction in systolic increments in $[Ca^{2+}]_c$ than was seen here [9, 30]. The negatively inotropic response to PDB seen in the present experiments can probably be attributed, therefore, at least in part, to a reduction in systolic increments in $[Ca^{2+}]_c$. That PKC activation was involved in the response is suggested by two lines of evidence. The first is the inactivity of PDD, a related phorbol ester that does not stimulate PKC [25]. The second is the mitigation of PDB-induced negative inotropism by means of the PKC inhibitor Ro31,8220. Nevertheless, PKC activation seems to be only partly responsible for the negatively inotropic response to PDB on account of the inability of a high concentration of Ro31,8220 to prevent such responses completely. A similar conclusion was reached by others in rat ventricular, myocardium [11], but using a different PKC inhibitor.

Possible involvement of PKC as a mediator of positively inotropic responses to phenylephrine is disputed (see above). Pretreatment of atria with PDB in the present experiments inhibited responses to both phenylephrine and isoprenaline, but not to ouabain or 4-aminopyridine. So far as phenylephrine and the phorbol esters are concerned this agrees with previous findings in rabbit hearts [6] and in rat isolated cardiac myocytes [39]. It is particularly significant that the selectively inhibitory effect of PDB against responses to adrenoceptor agonists in the present experiments was reversed by Ro31,8220. This appears to be the first report of a PKC inhibitor reversing an inhibitory action of a phorbol ester against the positively inotropic effects of phenylephrine. This constitutes strong evidence that the inhibitory action of PDB is mediated by PKC. It provides equally strong evidence that the positively inotropic effect of phenylephrine is not mediated by PKC, at least in rat atria. Instead, activation of PKC as a result of endogenous diacylglycerol formation following α-adrenoceptor stimulation may provide negative feedback regulation of the heart during intense activity of the sympathetic nervous system, as suggested previously [40]. Pretreatment of the atria with Ro31,8220 in the present experiments,

however, did not potentiate any of the responses to phenylephrine, casting doubt upon this last suggestion.

In several mammalian tissues the phosphorylation of α-adrenoceptors by means of PKC is known to reduce receptor affinity for both agonist and antagonist molecules, uncoupling receptors from intracellular second messenger systems and their resulting physiological responses [41–43], including those in the heart [6]. There appears to be no previous report, however, of a β-adrenoceptor-mediated cardiac response being blocked by pretreatment with a phorbol ester, although this is known to occur in smooth muscle [44]. In rabbit ventricular myocardium a slight potentiation of β-adrenoceptor-mediated inotropism has been found after treatment with a phorbol ester [6]. Why rat atria and rabbit papillary muscles should differ in this way is not known.

In summary, no evidence was found in the present experiments for an inotropically important activation of PKC during responses of rat atria to adrenoceptor agonists. Rather, the activation of PKC with a phorbol ester seems to inhibit the response to adrenoceptor agonists applied subsequently, and this was due to a reduction in the systolic increment in $[Ca^{2+}]_c$.

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