Effects of protein kinase C activation on cardiac repolarization and arrhythmogenesis in Langendorff-perfused rabbit hearts

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Introduction

Ventricular arrhythmias are still a major cause of death in western countries.1 Understanding arrhythmogenesis and development of safe antiarrhythmic drugs are important prerequisites to reduce excess mortality. To date, large multicenter studies could not demonstrate reduction of mortality with most of the currently available class I or III antiarrhythmic drugs. In some studies, excess mortality in the verum group even entailed early discontinuation of the trial.2–4 Implantable-cardioverter defibrillators (ICD) are now the treatment of choice for patients at high risk for sudden cardiac death. However, ICD therapy does not suppress occurrence of spontaneous ventricular arrhythmias, and painful defibrillation may occur. Moreover, implantation of ICDs is expensive and sometimes associated with major complications.

So far, less attention was paid to proarrhythmic effects of signalling pathways that potentially interact with cardiac ion channels. For instance, protein kinase A (PKA) and C (PKC) are downstream of G-protein-coupled signalling pathways that activate several effectors, such as ion channels in the heart.5

The PKC family consists of at least 10 isozymes. The most significant PKC family members for cardiac function belong to the subgroups PKC-α and PKC-β, and are activated by the presence of calcium and diacylglycerol. The ‘novel’ group PKC-δ and PKC-ε are activated by diacylglycerol with no requirement for calcium.6 These PKC isoforms are activated by membrane receptors coupled to Gq/G11 heterotrimeric G-proteins.6 Effects of PKC stimulation on whole heart electrophysiology are still not fully understood. Most findings originate from in vitro studies7–10 with sometimes confounding findings. In an ex vivo model activation of PKC during hypoxia facilitated occurrence of spontaneous ventricular fibrillation (VF), which was linked to opening of KATP channels.11 So far, little was known regarding proarrhythmic effects of PKC-activation in normal hearts.

The purpose of the present study was to investigate the effects of PKC stimulation on whole-heart electrophysiology and arrhythmogenesis in Langendorff-perfused hearts.

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Aims Cardiac arrhythmias are still a major cause of mortality in western countries. Currently available antiarrhythmic drugs are limited by a low efficacy and proarhythmic effects. The role of the protein kinase C (PKC) signalling pathway in arrhythmogenesis is still unclear. The goal of the present study was to test the effects of PKC stimulation on whole heart electrophysiology and its pro-/antiarrhythmic activity.

Methods and results Left ventricular (LV) action potential duration (APD 90%) was determined in 27 Langendorff-perfused rabbit hearts, using Tyrode solution plus the PKC agonist phorbol-12-myristate-13-acetate (PMA; 100 nM) alone (nine rabbits), Verapamil alone (n = 6), or PMA in combination with Verapamil (0.25 mg/L, six rabbits), or bisindolylmaleimide (0.5 μM, n = 6). Intermittent programmed extra-stimulation was performed to induce ventricular arrhythmias. Administration of PMA alone led to a significant shortening of repolarization (APD 90%, 157 ± 8 vs. 128 ± 5 ms, P < 0.05). Non-sustained ventricular fibrillation (VF) could be induced in seven out of nine animals. After perfusion of Verapamil (156 ± 6 vs. 169 ± 4 ms, P > 0.05) or bisindolylmaleimide, a selective inhibitor of PKC (136 ± 4 vs. 146 ± 4 ms, P > 0.05), PMA-induced shortening of repolarization could be inhibited, and induction of VF failed. Verapamil alone did not affect APD and VF could not be induced.

Conclusions Activation of PKC facilitates induction of VF, which is most likely due to a shortening of repolarization and a prominent calcium influx. These findings demonstrate involvement of the PKC-signalling pathway in arrhythmogenesis.

Keywords

Ventricular fibrillation; Protein kinase C; Verapamil; Repolarization; Monophasic action potentials; Arrhythmogenesis; Anti-arrhythmic drugs

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REFERENCES
Methods

All animal experiments conformed to the 'Position of the American Heart Association on Research Animal Use', adopted 11 November 1984.

Isolated rabbit heart preparation

The procedure has been described elsewhere. Briefly, 27 New Zealand White rabbits of either sex, weighing 2–3 kg, were anaesthetized with intravenous propofol (2%). Ten minutes before euthanasization rabbits received Buprenorphin–HCl 0.1 mg subcutaneously. After a median sternotomy, the heart was removed quickly and washed in cold Tyrode solution of the following composition (mM)—NaCl, 140; KCl, 5; CaCl 2, 2.2; MgCl 2, 1; NaHCO 3, 20; NaH 2PO 4, 0.33; glucose, 11.1; pH 7.4. Human albumin (0.040 mM) was added to the solution. The cut aortic stump was cannulated and the heart transferred to the Langendorff apparatus. Warmed, oxygenated (37°C, 95% O2, 5% CO2), modified Tyrode solution as described above was initiated. Base excess, pH, pO 2 and pCO2 were continuously measured and shown to be within a physiological range. Non-recirculating solution was perfused through the aorta at a constant flow of 25.8 ± 4.9 mL/min using a flow roller pump system (Pericor SF70/H33, Germany). The coronary flow was continuously measured with a glass flowmeter (Cole Parmer Instrument Company, Vernon Hills, IL, USA) positioned immediately above the retrogradely perfused aorta. The perfusion pressure in the cannulated aorta was kept at 80 mmHg during all experiments. Time interval from euthanization of rabbits and initiation of Krebs-Henseleit solution was 80 s.

Experimental protocol

All hearts were allowed to equilibrate for 20 min after instrumentation to confirm stability and viability. To ensure constant heart rates during baseline and post-arrhythmic recovery, pacing rate was set at 3.3 Hz (UHS 20, Biotronik, Germany). Hearts were stimulated through a pair of pacing electrodes (TME-60-Z, Osypka, Germany) located in the LV. The pacing threshold was stable for all protocols and with different pre-treatments. Epicardial monophasic action potentials (MAP) were recorded from the LV using Ag/AgCl contact MAP catheters (HSE, Germany) as described by Franz et al.13 Signals were amplified by an AC-Amplifier (Dieffenbacher AC 110, Germany) and stored on optical disks (optical disk DC-502, Pioneer Corp.) using a computer system (EPLab, Quinton Electrophysiology, USA). MAP duration (MAPD) was measured as the time from rapid depolarization to 90% repolarization.

After baseline MAPs were recorded during short periods of continuous ventricular pacing (BCL 3.3 Hz), hearts were perfused with Tyrode solution and phorbol-12-myristate-13-acetate (PMA; 100 nM) alone (nine rabbits) or in combination with Verapamil (0.25 mg/L, six rabbits) or bisindolylmaleimide (0.5 μM, n = 6). In order to evaluate effects of Verapamil alone, the protocol was repeated in another six rabbit hearts. To induce ventricular tachyarrhythmias sinus rhythm was interrupted by repetitive episodes of programmed extra-stimulation (BCL 3.3 Hz) at 1, 5, 10, 15, 20, and 25 min after start of perfusion with different agents. Inducibility of arrhythmias was tested during programmed stimulation at a basic cycle length of 3.3 Hz and coupling of one short extrabas. MAPs were always recorded immediately before application of programmed ventricular extra-stimulation.

Statistical analysis

Data are expressed as mean ± SEM. Basic comparative statistics were performed using a Student’s t-test for paired and unpaired data. ANOVA was applied where appropriate. A confidence level of 95% was considered statistically significant.

Results

Effects of phorbol-12-myristate-13-acetate on cardiac electrophysiology and inducibility of ventricular arrhythmias

Administration of Tyrode solution with PMA led to a significant shortening of LV repolarization [157 ± 6 vs. 128 ± 5 ms (mean MAPD minutes 1–25), P < 0.05]. Shortening of repolarization remained stable during the entire period of observation (Figures 1 and 2). In seven out of nine animals, non-sustained VF could be induced during programmed ventricular stimulation (Figure 3).

Effects of phorbol-12-myristate-13-acetate and verapamil on cardiac electrophysiology and inducibility of ventricular arrhythmias

In order to test whether calcium influx might be involved in PMA-induced shortening of repolarization as well as induction of VF, we added the calcium antagonist Verapamil to...
the solution. Interestingly, Verapamil fully antagonized PMA-induced shortening of repolarization \([156 \pm 6 \text{ vs. } 169 \pm 4 \text{ ms}, P > 0.05]\), and ventricular arrhythmias were no longer inducible (Figures 1 and 2).

In order to test whether Verapamil alone affects LV APD, we repeated experiments in additional six hearts. In comparison to baseline, Verapamil did not affect APDs \([145 \pm 9 \text{ vs. } 148 \pm 9 \text{ ms} (10 \text{ min perfusion of Verapamil}), 146 \pm 11 \text{ ms (20 min perfusion of Verapamil)}, 144 \pm 10 \text{ ms (25 min perfusion of Verapamil)}, P > 0.05)\) (Figure 2). Further, ventricular arrhythmias could not be induced.

**Effects of phorbol-12-myristate-13-acetate and bisindolylmaleimide on cardiac electrophysiology and inducibility of ventricular arrhythmias**

We tried to identify whether PMA-induced shortening of repolarization and induction of VF were related to PKC activation. Upon addition of bisindolylmaleimide, a selective inhibitor of PKC, PMA-induced shortening of repolarization was fully antagonized \([136 \pm 4 \text{ vs. } 146 \pm 4 \text{ ms}, P > 0.05; \text{Figures 1 and 2}]\). Induction of non-sustained VF was no longer possible.

**Discussion**

Marked shortening of ventricular APDs and induction of non-sustained VF during perfusion were the observed effects of PMA, an activator of PKC. Addition of Verapamil as well as the specific PKC-blocker bisindolylmaleimide successfully antagonized effects of PMA.

In the past, effects of PKC stimulation on cardiac electrophysiology were mainly linked to data obtained from isolated myocytes or *Xenopus* oocytes.\(^7\)\(^-\)\(^10\) PKC-stimulation could be shown to activate various effectors. The L-type calcium channels are regulated by Gq-linked receptors and associated PKC activation. The exact effect seems to be phosphorylation of L-type cardiac Ca\(^{2+}\) channels,\(^15\) which is associated with an increase in the Ca\(^{2+}\)-inward current. However, experiments utilizing direct activators of PKC have demonstrated time-dependent and biphasic effects on I\(_{\text{Ca,L}}\).\(^7\)\(^-\)\(^10\) In neonatal rat, ventricular myocytes and adult canine myocytes phorbol esters induced a marked calcium-influx within the first 20 min of perfusion, whereas on later stimulation the opposite effects could be observed.\(^8\)\(^-\)\(^10\) In the present study sustained shortening of repolarization and induction of VF could be observed during the entire period of observation (25 min). Further, non-sustained VF could be induced by programmed ventricular stimulation.

In a recent study PKC-induced facilitation of VF could be demonstrated in Langendorff-perfused rabbit hearts subjected to hypoxia and re-oxygenation.\(^11\) Interestingly, adding Glibenclamide, a potent K\(_{\text{ATP}}\)-channel blocker, to the PKC-activator, VF was successfully inhibited. These results demonstrate that the proarrhythmic effect of PKC activation is manifest under conditions in which myocardial ATP concentration is known to be reduced. However, the mechanism underlying PKC-induced shortening of APD in normal hearts seems to be different, since depletion of ATP usually does not occur in normal hearts. Thus, involvement of K\(_{\text{ATP}}\) channels is rather unlikely.

Findings of the present study indicate a potential antiarrhythmic effect of Verapamil during PKC-activation. Interestingly, Verapamil not only suppressed induction of non-sustained VF, it also prevented PMA-induced shortening of repolarization. The question arises, whether reduction of cellular calcium concentration or direct effects of Verapamil on major repolarizing potassium channels are responsible for the antiarrhythmic activity. In order to exclude direct effects on repolarizing potassium channels, we determined APDs during perfusion of Verapamil alone. Not surprisingly, Verapamil did not affect APDs. Similar results have already been reported by Chorro et al.\(^16\) Based on these results major direct effects of Verapamil on repolarizing potassium channels seem to be rather unlikely. A second hypothesis is linked to interaction between intracellular calcium, calcium-dependent ion channels, and Verapamil. In recent studies, intracellular Ca\(^{2+}\) overload mediates activation of repolarizing ion channels, e.g. calcium-dependent-potassium\(^17\)\(^-\)\(^18\) and/or chloride channels\(^17\)\(^-\)\(^19\) could be shown. Both channels are present in rabbit hearts,\(^20\) however, their exact role has not been determined yet.

A third hypothesis is based on the direct effects of Verapamil on intracellular calcium concentration. Chudin et al.\(^21\) could demonstrate a dual effect of cytoplasmic-free calcium on the APD. On the one hand, a large Ca\(^{2+}\) transient tends to shorten APD by increasing Ca\(^{2+}\)-dependent inactivation of the L-type calcium channel. However, this effect on APD is counteracted by enhanced inward Na\(^+\)-Ca\(^{2+}\) exchange and non-specific calcium-activated currents. Another focus of their study was to elucidate the mechanisms underlying transition from ventricular tachycardia (VT) to VF. Interestingly, transition from VT to VF started with an unusually large calcium transient, which was caused by spontaneous calcium release. This resulted in a substantial prolongation of the action potential, due to an increase in inward components of I\(_{\text{Na,L}}\), I\(_{\text{Na,Ca,Ca}}\). The result was a marked shortening of the subsequent diastolic interval, which also shortened the duration of the subsequent action potential. Conversely, the short diastolic interval and altered action potential affected the intracellular calcium transient of the next beat, which further modifies the action potential through its feedback on...
calcium-sensitive currents. Similar mechanisms are conceivable for marked abbreviation of APDs during PKC-activity in normal hearts. Further studies are required to confirm this hypothesis.

Antiarrhythmic effects of Verapamil could also be confirmed in acute myocardial infarction and during hypoxia and reoxygenation. In open chest pigs with acute myocardial infarction, administration of Verapamil successfully suppressed occurrence of VF. Similar results were evident in an ischaemia/reperfusion model. However, antiarrhythmic effects of Verapamil are limited by a significant increase of defibrillation threshold and haemodynamic deterioration. Therefore, Verapamil is not a first line antiarrhythmic drug.

Direct effects of PKC on other than L-type calcium channels are still a matter of debate. In order to elucidate, whether additional channels are involved in PKC-induced shortening of repolarization and induction of VF, we performed additional experiments. As shown in a recent in vitro study, both agonistic and antagonistic effects of PKC on one of the two major repolarizing ion currents, the slow component of the delayed rectifier potassium current (I_Ks), could be demonstrated. PKC-activation first increased and later decreased the current through I_Ks, with a peak of I_Ks 20 min after activation of PKC and a decrease of I_Ks after 60 min. In our model, we tried to inhibit I_Ks by perfusing HMR 1556, a selective blocker of I_Ks. Unfortunately, 5 min after starting the perfusion, progressive pump failure occurred in all hearts (data not shown). These negative effects of HMR 1556 might be due to DMSO, a solution required for dissolving HMR 1556. We admit that this might be a limitation of the study.

Involvement of the PKC pathway in arrhythmogenesis could be confirmed by suppression of PMA-induced shortening of repolarization and absence of VF by bisindolylmaleimide, a specific blocker of PKC.

In accordance with a significant shortening of repolarization, the present study also highlights the proarrhythmic potential of PKC activation. In seven out of nine experiments, induction of VF was possible. Arrhythmogenesis during PKC stimulation might be caused by a significant intracellular calcium-overload triggering VF during application of short, coupled extrabeats. This hypothesis is supported by the observation that Verapamil completely blocked induction of VF, although Chorro et al. demonstrated a shortening of the wavelength and increased frequency of VF with Verapamil, which would potentially stabilize VF. These findings were also confirmed with the calcium channel-blocker Nifedipine. Conversely, Chudin et al. described suppression of transition from VT to VF, when decreasing the sensitivity of calcium-sensitive currents. In their study, spontaneous calcium release acts as a gain-enhancing mechanism between intracellular calcium and calcium-sensitive currents. The question arises, whether similar mechanisms might underlie the antiarrhythmic activity of Verapamil in the present study. Further studies are warranted to clarify this.

Regarding induction of VF during PKC-activation the following mechanism is conceivable: Aberrant calcium fluxes may result from both inappropriate sarcoplasmic reticulum calcium release and from excessive calcium influx via L-type calcium channels. Latter can be mediated via PKC activation. Both calcium channels and the ryanodine receptors in the endoplasmic reticulum are involved in the dysregulation of calcium signalling that may contribute to cardiac arrhythmias. It could be shown that excessive calcium influx via the L-type calcium channel together with early extrabeats may initiate plasmamembrane depolarization in cardiomyocytes. These, so called, early or delayed after-depolarizations are able to trigger polymorphic ventricular tachycardias.

So far, no data are available demonstrating a correlation between increased PKC-activity and an increased risk for sudden cardiac death in humans. Merely, in mice with transgene expression of PKC-β sudden death frequently occurred, which was associated with marked abnormalities in the regulation of intracellular calcium.

Clinical implication

Although other mechanisms might be operative, the data presented here indicate that direct targeting of intracellular kinases is feasible and might have strong antiarrhythmic potency. However, several studies have shown side-effects of antagonizing PKC-activity. It is well known that the main pathway of the preconditioning effect is mediated by activation of PKC and opening of mitochondrial K_ATP channels and the prevention of DNA fragmentation in myocytes. The process of reperfusion leads to an excessive accumulation of free radicals, that are believed to damage the myocardium. Using free radical scavengers before reperfusion, a significant reduction in infarct size can be achieved. Unfortunately, perfusion of Chelerythrine, a non-selective PKC inhibitor, fully abolished effects of free radical scavengers.

However, antagonizing PKC also exhibits beneficial effects on cardiac haemodynamics. As shown by Wang et al., short periods of fast ventricular pacing are mediating depression of contractile function via stimulation of a Ca^{2+} / PKC-dependent signalling mechanism. Exposure to PMA, an activator of PKC, significantly inhibited cell shortening, whereas Chelerythrine, a non-selective PKC inhibitor, prevented pacing-induced inhibition of cell shortening.

In order to develop effective antiarrhythmic drugs targeting PKC, major side-effects like prevention of preconditioning or depression of cardiac performance need to be excluded.

Limitations

One limitation of our study is the fact that we have not studied effects of PMA, Verapamil, and bisindolylmaleimide on a cellular level. Therefore, we are not able to exactly describe the mechanisms underlying PKC-induced shortening of repolarization and induction of VF. Further, without determining PKC expression/activity, calcium flow and cellular calcium concentrations interactions between PKC, intracellular calcium, and ion channel function cannot be fully explained. In addition, transmural differences in the functional effects of PKC activation on potassium channels might have played a role in induction of VF. In our study we did not evaluate transmural refractoriness and expression of PKC, so we are not able to draw any final conclusion. Further, we did not test effects of bisindolylmaleimide alone on APD. This is another limitation, because bisindolylmaleimide might directly affect additional signalling pathways and/or ion channels.
Conflict of interest: none declared.

References


