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SPECIAL REPORT

Protein kinase C inhibitors enhance endothelin-1 and attenuate vasopressin and angiotensin II evoked $[Ca^{2+}]_i$ elevation in the rat cardiomyocyte

Yanjun Xu, L. Sandirasegarane & [1]Venkat Gopalakrishnan

Cardiovascular Risk Factor Reduction Unit (CRFRU), Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, SK., S7N OWO, Canada

Primary cultures of neonatal rat cardiomyocytes were pretreated for 16 h with either nonselective (staurosporine, 100 nM) or selective (NPC15437, 20 μM) protein kinase C (PKC) inhibitors. These inhibitors did not affect the basal cytosolic free calcium, $[Ca^{2+}]_i$, level (106 ± 12 nM) as determined by fura-2 fluorescence methodology. Both agents significantly enhanced the maximal $[Ca^{2+}]_i$ responses to endothelin-1 (ET-1) and attenuated the peak $[Ca^{2+}]_i$ responses to arginine vasopressin and angiotensin II. They did not alter the EC_{50} values of any of these agonists. Since depletion of $[Ca^{2+}]_o$ led to only partial attenuation of the enhanced response to ET-1 in the treatment groups, it is likely that PKC inhibition results in an exaggerated intracellular mobilization of Ca^{2+} to ET-1. It is concluded that PKC modulates agonist(s)-evoked intracellular Ca^{2+} mobilization and that the nature of regulation is governed by the agonist.

Keywords: Rat cardiomyocyte; fura-2 fluorescence; cytosolic free Ca^{2+}; protein kinase C; staurosporine; vasopressin; angiotensin II; endothelin-1

Introduction Several studies have demonstrated the presence of receptors for vasoactive peptides such as angiotensin II (AII) and endothelin-1 (ET-1) in cardiomyocytes. It was not known if receptors for arginine vasopressin (AVP) were also present on these cells to account for its direct action on the heart. Recently, we have shown that the neonatal rat cardiomyocyte does express V_1 subtype receptors for AVP which are linked to Ca^{2+} mobilization (Xu & Gopalakrishnan, 1991). These receptors are coupled to activation of phosphoinositidase C which stimulates the formation of diacylglycerol, an activator of protein kinase C (PKC). Because PKC could regulate cardiac function, we sought to examine its role in peptide agonist(s)-evoked alterations in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). Previous studies in non cardiac tissues have utilized phorbol esters which are known to activate PKC (Chardonnens *et al.*, 1990; Simonson & Dunn, 1992; Iijima *et al.*, 1992). However, prolonged incubation with phorbol esters causes downregulation of PKC activity, making studies with these esters difficult to interpret. Therefore, in the present study, we have taken a more direct approach by pretreating the cells with highly potent and selective PKC inhibitors to elucidate the regulatory role of PKC on resting and peptide agonist(s)-evoked alterations $[Ca^{2+}]_i$ using primary cultures of neonatal rat cardiomyocyte.

Methods The methodology for dispersing neonatal rat cardiomyocytes, maintaining these cells in primary culture, fura-2 loading and $[Ca^{2+}]_i$ measurement procedures have been described (Xu & Gopalakrishnan, 1991). In the present study, we added either staurosporine (STS) or 2,6-diamino-N-([1-(1-oxotridecyl)-2piperidinyl]methyl)hexanamide (NPC 15437) to culture flasks so that their final concentrations in the medium were 100 nM and 20 μM respectively. These agents were maintained in culture for 16 h before the experiment. The $[Ca^{2+}]_i$ levels at rest, as well as at the maximal increase evoked by the addition of agonists (AVP, AII and ET-1) were determined. The responses to all the three agonists were tested on the same day with both STS-treated and control groups of cells for comparison. Select experiments for ET-1-evoked increases in $[Ca^{2+}]_i$ were conducted in the absence of extracellular Ca^{2+} with 1 mM EGTA being present in the buffer. Statistical significance of differences between means was estimated by ANOVA.

Materials STS was obtained from Calbiochem (San Diego, CA, U.S.A.). NPC 15437 was a gift from Nova Pharmaceutical Corporation (Baltimore, MD, U.S.A.). Fura-2AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). A stock concentration of STS (1 mM) was prepared in dimethylsulphoxide and subsequent dilutions were made in either culture or incubation medium. AVP, AII and ET-1 were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.).

Results There were no differences in the basal $[Ca^{2+}]_i$ levels between control (106 ± 12 nM) and treated (STS, 113 ± 11 nM; NPC 15437, 103 ± 9 nM) groups. The effect of STS pretreatment on the responses to AVP, AII and ET-1 are shown in Table 1. All three peptides evoked concentration-dependent increases in $[Ca^{2+}]_i$ above the basal levels. The order of potency, as determined by the EC_{50} values, for the evoked maximal $[Ca^{2+}]_i$ values was similar for the three peptides. The EC_{50} values of these agonist(s) were not significantly altered by STS pretreatment. The order of efficacy, as determined by the maximal increase in peak $[Ca^{2+}]_i$ response induced by each agonist, was AII > AVP > ET-1. STS pretreatment led to large reductions ($P < 0.01$) in the evoked maximal $[Ca^{2+}]_i$ responses to both AVP and AII. In contrast, the evoked maximal $[Ca^{2+}]_i$ response to ET-1 was significantly elevated ($P < 0.01$) in the STS-treated cells (Table 1). Removal of $[Ca^{2+}]_o$ led to significant decreases ($P < 0.05$) in the basal $[Ca^{2+}]_i$ values for both control group (from 107 ± 6 to 83 ± 5 nM) and STS (from 115 ± 10 to 87 ± 7 nM) pretreated cells. However, no significant differences were observed in the percentage increases in peak $[Ca^{2+}]_i$ (above the basal) values to ET-1 (25 nM) either in the presence (239 ± 15%) or absence

[1] Author for correspondence.

Table 1 Analyses of the effect of staurosporine (STS) pretreatment (100 nM) on arginine vasopressin (AVP), angiotensin II (AII) and endothelin-1 (ET-1)-evoked cytosolic free $[Ca^{2+}]$ increase in neonatal rat cardiomyocyte (25°C)

Agonists	EC_{50} (nM) Control	STS	$[Ca^{2+}]_i$ – Control	E_{max} (nM) STS
AVP	10 ± 2	12 ± 3	245 ± 30	112 ± 5**
AII	12 ± 4	9 ± 2	960 ± 75	265 ± 16**
ET-1	7 ± 1	11 ± 4	175 ± 11	298 ± 21**

Each value is mean ± s.e.mean of 7 separate determinations. Basal $[Ca^{2+}]_i$ values (in the absence of agonist stimulation) were not significantly different between control (106 ± 12 nM) and STS (113 ± 11 nM) pretreated cells. **$P<0.01$ compared to control values.

(219 ± 11%) of $[Ca^{2+}]_o$. NPC 15437 (20 μM) pretreatment also resulted in a qualitatively similar reduction in responses to AVP and AII and a significant increase in the $[Ca^{2+}]_i$ response to ET-1 (Figure 1).

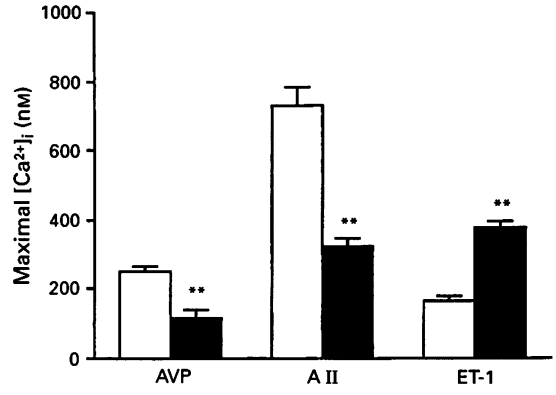

Figure 1 The comparison of peak $[Ca^{2+}]_i$ response to a fixed concentration of 25 nM of arginine vasopressin (AVP), angiotensin II (AII), or endothelin-1 (ET-1) in the dispersed neonatal rat cardiomyocyte in the presence (■) or in the absence (□) of the protein kinase C (PKC) inhibitor, NPC-15437 (20 μM). Each column represents mean ± s.e.mean (vertical bars) of 5 separate experiments performed with different batches of cardiomyocytes. Basal $[Ca^{2+}]_i$ values (in the absence of agonist stimulation) were not significantly different between control group (106 ± 12 nM) and NPC (103 ± 9 nM) pretreated cells. There was a residual response of 20 ± 4 nM increase above the basal $[Ca^{2+}]_i$ values for AVP in the NPC pretreated group.
**Denotes $P<0.01$ compared to control group.

Discussion In addition to comparing the $[Ca^{2+}]_i$ responses to AVP, AII and ET-1 in the neonatal rat cardiomyocytes for the first time, this study demonstrates a qualitatively differential effect of inhibition of PKC on the responses to these peptides: the inhibitors of PKC, STS and NPC 15437, enhanced $[Ca^{2+}]_i$ response to ET-1 and attenuated the responses to AVP and AII. STS is a highly potent but nonselective inhibitor of several kinases and binds to their catalytic moiety. Although several studies in the past have used this agent to elucidate the role of PKC, its selectivity in blocking PKC has recently been questioned (Kageyama *et al.*, 1991). In order to address this issue, we also used a selective inhibitor of PKC, NPC 15437, which blocks the regulatory subunit of PKC, a site at which diacylglycerol binds to activate the enzyme (Sullivan *et al.*, 1992). Neither STS nor NPC 15437 pretreatment *per se* affected fura-2 fluorescence. The observation that similar results were obtained with two inhibitors of PKC that act on different sites of the enzyme adds credibility to the conclusion that the altered responses to the peptide agonists were a consequence of inhibition of PKC.

The $[Ca^{2+}]_i$ response to ET-1 in the presence and absence of inhibition of PKC has not been reported previously for cardiomyocytes. In vascular smooth muscle tissue, ET-1-evoked vasoconstriction was reversed by H-7, a nonselective PKC inhibitor (Sugiura *et al.*, 1989). An increase in the prolonged secondary phase of Ca^{2+} mobilization was demonstrated previously with a fixed high concentration of ET-1 in either STS or phorbol ester pretreated rat mesangial single cells (Iijima *et al.*, 1991; Simonson & Dunn, 1991). Neither of these studies in non-cardiac tissue examined the role of $[Ca^{2+}]_o$. The present study in cardiomyocytes has shown that $[Ca^{2+}]_i$ response to ET-1 in the STS group was attenuated only to a small extent by removal of $[Ca^{2+}]_o$. Therefore, it is likely that the exaggerated $[Ca^{2+}]_i$ response to ET-1 was due primarily to mobilization of Ca^{2+} from intracellular stores. Recently, it has been shown that enhanced PKC activity and reduced receptor density for ET-1 seen in diabetic renal glomerular mesangial cells could be normalized by the inclusion of a PKC inhibitor (Awazu *et al.*, 1991). Thus, on the basis of our results in cardiomyocytes, it seems reasonable to suggest that cell surface ET-1 receptor is tonically downregulated by PKC and that STS or NPC 15437 abolishes this negative feed back modulation by PKC thereby resulting in an exaggerated $[Ca^{2+}]_i$ response to ET-1.

The observation of diminished $[Ca^{2+}]_i$ responses to both AVP and AII in the cells pretreated with PKC inhibitors is consistent with the previous report of decreased $[Ca^{2+}]_i$ responses to these two agonists when PKC is downregulated by prolonged incubation with tumour promoting phorbol ester (Chardonnens *et al.*, 1990). However, the mechanism underlying the diminished response to these agonists needs further study.

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