Phorbol ester inhibition of vasopressin-induced calcium efflux from cultured rat aortic myocytes

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The effect of the protein kinase C activator TPA was investigated on AVP-induced ^{45}Ca release from rat aortic myocytes. In the nanomolar range TPA, but not 4β-phorbol, reduced the brief ^{45}Ca efflux produced by AVP in the presence or in the absence of extracellular calcium. The maximal effect of TPA was to abolish the response to a half maximally active concentration of AVP, and to reduce by 50% the maximal response to the hormone. These results suggest that protein kinase C activation can exert a negative control on the early AVP-induced calcium mobilization in vascular smooth muscle.

Phorbol ester *Protein kinase C* *Vasopressin* *$^{45}Ca^{2+}$ efflux* *(Vascular smooth muscle)*

1. INTRODUCTION

In vascular smooth muscle cells, vasopressin and other calcium mobilizing agonists stimulate the hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate [1,2] and produce a transient increase in the cytoplasmic concentration of free Ca^{2+} [2,3]. The phosphoinositide hydrolysis yields two putative second messengers, inositol 1,4,5-trisphosphate, which can release Ca^{2+} from an intracellular store in vascular smooth muscle [4] and diacylglycerol, which activates protein kinase C.

The physiological role of protein kinase C is still largely unknown in vascular smooth muscle. It has been reported that the tumor-promoting phorbol ester TPA, which is able to substitute for endogenous diacylglycerol in activating protein kinase C, potentiates contractions of vascular smooth muscle produced by entry of Ca^{2+} [5], and it has been suggested that this mechanism may account for the persistence of tonic contractions after an initial rise of cytoplasmic free Ca^{2+} concentration [5]. In other tissues, TPA although increasing the effect of calcium [6] inhibits cell responses mediated by the inositol trisphosphate-calcium pathway [7]. Experiments were therefore designed to investigate the effect of TPA on calcium release from vascular smooth muscle cells elicited by [Arg]^8-vasopressin (AVP). The experiments were made on primary cultures of rat aortic myocytes, which possess vasopressin receptors and contract upon the addition of AVP [8]. ^{45}Ca efflux from myocytes preloaded with the radioisotope was measured, since a temporal correlation between phosphoinositide hydrolysis, calcium efflux and the onset of contraction has been reported in vascular smooth muscle [9].

Abbreviations: AVP, [Arg^8]-vasopressin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PMT-AVP, [(β-mercapto-β,β-cyclopentamethylene propionic acid)^1, (*O*-methyl)Tyr^2,Arg^8]-vasopressin

2. MATERIALS AND METHODS

Confluent primary cultures of thoracic aortic myocytes from 10–12-week-old female Wistar rats

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were obtained as in [10] and used 8–10 days after plating. Unless otherwise mentioned, the experiments were carried out at 37°C and pH 7.4 with 1.8 mM calcium in serum-free culture medium (Eagle's minimum essential medium, Eurobio). In each experiment cells obtained from the same cell suspension, plated in 60 mm petri dishes were used, each dish containing about 3.5×10^6 cells. Cells were preincubated for 3 h with ^{45}Ca (Amersham) (3 μCi per μmol Ca). TPA (Sigma) or 4β-phorbol (Peninsula laboratories) were added to the test dishes 15 min before the end of ^{45}Ca loading. As these compounds were dissolved in dimethyl sulfoxide, this solvent was also added to the control dishes at the same final concentration of 0.05%. Efflux was monitored by liquid scintillation counting of ^{45}Ca collected in the bathing solution, which was replaced every 10 s. A steady-state efflux rate was established after an initial 2 min wash period. AVP (Sigma) was then added to successive rinses of medium. Preliminary experiments showed that addition of $10^{-7} \times M$ AVP for 20 s was sufficient to induce a complete efflux response whereas the efflux induced by lower concentrations of AVP could be slightly prolonged by addition of agonist beyond 20 s.

In experiments requiring a V_1 receptor antagonist, PMT-AVP (Peninsula Laboratories) was added simultaneously with AVP.

Other efflux experiments were carried out either in a saline solution (millimolar composition: 143 Na^+, 5.84 K^+, 1.25 Ca^{2+}, 1.19 Mg^{2+}, 125.2 Cl^-, 25 HCO_3^-, 1.14 $H_2PO_4^-$, 1.19 SO_4^{2-}, 10 glucose) or in the same solution with no added calcium and 1 mM EGTA.

3. RESULTS

As illustrated in fig.1, AVP induced a transient increase in ^{45}Ca efflux. The onset of this effect was rapid, a peak value was reached in 20–30 s after addition of AVP and the efflux rate returned to baseline in 80–100 s. The results in table 1 show that AVP-induced ^{45}Ca efflux was concentration-dependent in the range 10^{-9}–10^{-7} M, the half-maximal effective concentration of AVP being about 10^{-8} M.

^{45}Ca efflux induced by AVP could be produced in the presence or absence of 1.25 mM extracellular calcium, and was even greater in the latter case (fig.1).

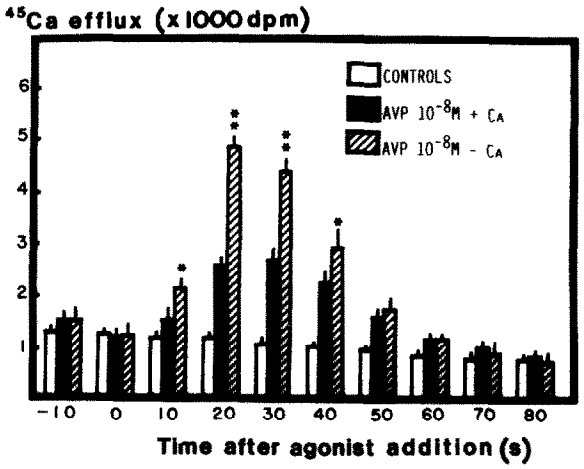

Fig.1. Time course of AVP-induced ^{45}Ca efflux from rat aortic myocytes in primary cultures and influence of extracellular calcium concentration. Prior to drug addition efflux was carried out during 2 min in saline solutions containing either 1.25 mM Ca or no added calcium and 1 mM EGTA (see section 2). Columns represent ^{45}Ca released into the medium during 10 s intervals. In this representative experiment, results are means of 3–5 determinations, each corresponding to one petri dish, vertical lines represent SE. $*P<1\%$, $**P<0.1\%$: significance of difference between treatments evaluated by two-way analysis of variance.

A V_1 receptor antagonist, PMT-AVP, at 10^{-6} M abolished ^{45}Ca efflux induced by 10^{-8} M AVP and decreased the effect of 10^{-7} M AVP by about 75% (table 2).

Table 2 also shows that 10^{-7} M TPA did not

Table 1

Concentration dependence of AVP-induced ^{45}Ca efflux from cultured rat aortic myocytes

AVP concentration (M)	Total ^{45}Ca released (dpm)[a]	% of maximum[b]
0	5472 ± 336	0 ± 3
10^{-9}	6226 ± 242	6 ± 2
10^{-8}	12765 ± 337	54 ± 3
10^{-7}	17017 ± 1817	86 ± 14
10^{-6}	18928 ± 1480	100 ± 11

[a] Sum of dpm collected during 1 min following addition of AVP; values are means ± SE of 4–6 determinations
[b] Values deduced from means in middle column

Table 2

Effect of PMT-AVP, TPA and 4β-phorbol on AVP-induced ^{45}Ca efflux from cultured myocytes

Treatment	Total ^{45}Ca released (dpm)[a]	% of maximum[b]
Control	6560 ± 420	0 ± 3
TPA (10^{-7} M)	7169 ± 414	4 ± 3
AVP (10^{-8} M)	11826 ± 1009	38 ± 7
+ PMT-AVP (10^{-6} M)	6449 ± 736	−1 ± 5
+TPA (10^{-7} M)	6365 ± 314	−1 ± 2
+ 4β-phorbol (10^{-5} M)	12778 ± 556	45 ± 4
AVP (10^{-7} M)	20420 ± 525	100 ± 4
+ PMT-AVP (10^{-6} M)	10025 ± 847	25 ± 6
+ TPA (10^{-7} M)	14737 ± 1071	59 ± 8
+ 4β-phorbol (10^{-5} M)	24162 ± 4566	127 ± 33

[a] Values obtained as in table 1 are means ± SE of 3–5 determinations

[b] Values relative to mean total ^{45}Ca released by 10^{-7} M AVP

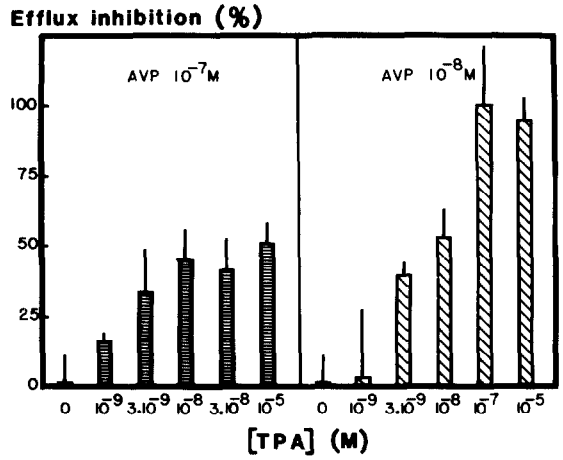

Fig.2. Concentration-dependent inhibition by TPA of ^{45}Ca efflux from cultured myocytes stimulated either by 10^{-7} M AVP (left) or 10^{-8} M AVP (right). Total ^{45}Ca released was calculated as in table 1 and inhibition percentages deduced. 0% inhibition corresponds to AVP-induced efflux in the absence of TPA; 100% is equivalent to the non-stimulated efflux level. Results are means of 3–7 determinations, vertical lines represent SE.

modify the basal ^{45}Ca efflux from resting cells, but reduced AVP-stimulated ^{45}Ca efflux. Unlike TPA, 4β-phorbol (an analog of TPA which does not activate protein kinase C) at concentrations up to 10^{-5} M did not decrease ^{45}Ca efflux induced by either 10^{-8} or 10^{-7} M AVP (table 2).

Fig.2 shows the concentration dependence of the TPA effect on ^{45}Ca efflux induced by 10^{-7} and 10^{-8} M AVP. In both cases TPA was effective in the 10^{-9} M range, with half-maximal effects close to 3×10^{-9} M. The effect of the lower concentration of AVP was abolished by 10^{-7} M TPA, but even higher concentrations of TPA (up to 10^{-5} M) only decreased by 50% the effect of the higher concentration of AVP. This inhibition remained partial in the absence of extracellular calcium (not shown).

4. DISCUSSION

The findings that AVP stimulated ^{45}Ca efflux from aortic myocytes in primary culture, that this effect was inhibited by a V_1 antagonist, and that it could be produced in the absence of extracellular calcium, are consistent with the view that the stimulation of a V_1 receptor by AVP induced the release of ^{45}Ca from one or more intracellular stores, followed by an efflux from the cell. This is also consistent with the rise in cytoplasmic calcium concentration seen in quin 2 fluorescence experiments [2,3] and the decrease in ^{45}Ca content elicited by AVP [1] in other vascular smooth muscle cells. The fact that ^{45}Ca efflux was greater in the absence than in the presence of extracellular calcium might further indicate that an influx of non-radioactive isotope also takes place in the presence of extracellular calcium, producing an isotopic dilution of intracellular ^{45}Ca and a smaller efflux of radioisotope.

The increase in ^{45}Ca efflux elicited by AVP ceased rapidly in the continuous presence of the hormone, even at concentrations not producing a maximal effect. Thus, ^{45}Ca efflux returned to baseline before the quantity of radioisotope which could be released from the cells was exhausted. The question then arose of the possible implication of protein kinase C activation in the termination of AVP-induced calcium efflux.

AVP-stimulated ^{45}Ca efflux was inhibited after preincubation of the myocytes with TPA. The phorbol ester was effective in a concentration range (10^{-9}–10^{-7} M) at which it was reported to stimulate protein kinase C in cells [11] and the inhibitory effect of TPA was not produced by a TPA analog 4β-phorbol, which does not activate protein kinase C [12]. At concentrations associated with activation of protein kinase C, TPA alters various cell responses mediated by the inositol trisphosphate pathway [7,13]. The mechanism by which protein kinase C activation may inhibit AVP-induced calcium mobilization in the present study is not known. Phosphorylation of the receptor itself, modifying its affinity for agonists (in the case of adrenoreceptors see [14]), or phosphorylation of the protein coupling the receptor to phospholipase C may inhibit phosphoinositide hydrolysis and subsequent calcium release.

Whatever the mechanism by which activation of protein kinase C produces an inhibition of AVP induced ^{45}Ca efflux, the present results show that TPA can abolish the effect of a half-maximally effective concentration of AVP, whereas even high concentrations of TPA could only reduce by half the efflux elicited by a maximally effective concentration of AVP. This TPA resistant component of AVP response cannot be due to an entry of extracellular calcium at high concentrations of agonist, since the inhibition remained partial in the absence of external calcium.

The early phasic increase in cytoplasmic concentration of free Ca^{2+} [2,3] and in calcium efflux (this study) elicited by AVP in aortic myocytes is followed by a sustained contraction. It has been suggested that the persistence of a tonic contractile response of the vessel after the phasic effect of the hormone on intracellular calcium mobilization could be the consequence of protein kinase C activation by diacylglycerol, since TPA can elicit a late and sustained contraction in isolated rat aorta [5]. The results reported here, showing an inhibitory effect of TPA on AVP-induced calcium efflux, suggest that protein kinase C activation can in addition inhibit calcium mobilization produced by AVP and may participate in the negative retrocontrol of this phenomenon, which must take place to explain its rapid cessation after addition of AVP. However, the finding that TPA did not completely abolish calcium efflux induced by AVP at concentrations producing a maximal effect might further suggest that protein kinase C activation may not be the sole mechanism responsible for this retrocontrol.

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