Effect of the phorbol ester TPA on PTH secretion

Evidence for a role for protein kinase C in the control of PTH release

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Parathyroid hormone (PTH) secretion is stimulated by low extracellular calcium (Ca^{2+}) in association with a reduction in cyosolic Ca^{2+}, indicating that this cell type does not conform to classical models of stimulus–secretion coupling. We used the phorbol ester TPA (12-O-tetradecanoyl phorbol 13-acetate), which directly activates protein kinase C, to investigate the possible role of this enzyme in the unusual secretory properties of the parathyroid cell. TPA causes a dose-dependent stimulation of PTH release inhibited by high extracellular Ca^{2+} ($EC_{50} = 10$ nM) but has relatively little effect on secretion stimulated by low Ca^{2+}. This effect was mimicked by the β4-isomer of phorbol 12,13-didecanoate which also activates kinase C, but not by the α4-isomer, which has no effect on this enzyme. TPA does not modify cellular cAMP or cytosolic Ca^{2+} in the parathyroid cell indicating that its effects on PTH secretion are not mediated indirectly via changes in these second messengers. These results suggest that inhibition of PTH release at high Ca^{2+} might be related to a reduction in protein kinase C activity which can be overcome when the enzyme is directly activated by TPA.

PTH release *Phorbol ester* *Cytosolic Ca^{2+}* *cAMP*

1. INTRODUCTION

Recent studies have emphasized the unusual secretory properties of the parathyroid cell. At very low extracellular Ca^{2+} concentrations ($< 10^{-7}$ M), maximal rates of hormonal secretion are maintained despite cytosolic Ca^{2+} concentrations of less than 100 nM [1]. Moreover, as extracellular Ca^{2+} is increased, the cytosolic Ca^{2+} rises to 600–700 nM in association with a progressive inhibition of PTH release [1]. Finally, dopamine, which stimulates PTH secretion by 2–4-fold and elevates cellular cAMP by 20–40-fold [2], has no effect on cytosolic Ca^{2+} [1]. These characteristics of the parathyroid cell cannot be accounted for by classical models of stimulus-secretion coupling, in which elevations in cytosolic Ca^{2+} are linked to stimulation of hormonal secretion [3]. Accumulating evidence has suggested that protein kinase C, which is activated by Ca^{2+}, phospholipids, and diacylglycerol, may also play an important role in exocytosis [4]. We therefore employed the phorbol ester TPA (12-*O*-tetradecanoyl phorbol 13-acetate), an activator of protein kinase C which is capable of stimulating secretion independent of changes in cytosolic Ca^{2+} [5], as a probe to assess the potential role of this enzyme in PTH secretion.

2. MATERIALS AND METHODS

Dispersed bovine parathyroid cells were prepared as previously described [6]. Cells prepared in this fashion show >95% exclusion of Trypan blue, maintain high cellular ratios of potassium to sodium [7], release hormone linearly for several hours, and are responsive to Ca^{2+} and diverse hormonal factors in a manner similar to

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that observed in vivo. For determination of PTH release or cAMP accumulation, cells were incubated at 37°C for varying times in Eagle's MEM (Earle's salts, without bicarbonate, Ca^{2+} or Mg^{2+}) supplemented with 0.02 M Hepes (pH 7.47), 0.2% bovine serum albumin (BSA) and 0.5 mM $MgSO_4$ ('standard medium') as well as the concentrations of $CaCl_2$ and other additives indicated below. PTH was measured in supernatant samples after sedimentation of the cellular pellet at 100 × *g* for 2 min, using an antiserum raised against bovine PTH (GW-1), which recognizes the intact hormone and C-terminal fragments of PTH [6]. cAMP was extracted from the cells with TCA and measured by radioimmunoassay as described previously [6]. TPA and other additives used in these experiments had no effect on the radioimmunoassays for cAMP and PTH.

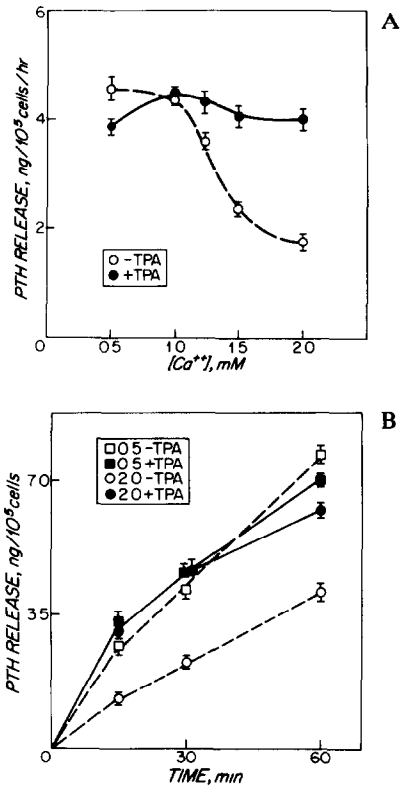

Fig.1. (A) Effect of TPA on Ca^{2+}-regulated PTH release. Parathyroid cells (1×10^6/ml) were incubated at varying extracellular Ca^{2+} concentrations (●) with or (○) without 10^{-6} M TPA for 60 min at 37°C as described in section 2. PTH was determined by radioimmunoassay and points represent the mean ± SEM for 9 determinations in 3 experiments. (B) Time course for effect of TPA on PTH release. Cells were incubated at 37°C with 0.5 (□, ■) or 2 (○, ●) mM Ca^{2+} with (■, ●) or without (□, ○) 10^{-6} M TPA for the times indicated. PTH release was determined as above. Points are the mean ± SEM for 9 determinations in 3 experiments.

For determination of cytosolic Ca^{2+}, parathyroid cells were loaded with QUIN-2 as described [1], washed and resuspended in a solution containing 0.025 M Hepes (pH 7.45), 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 g/l dextrose, and 1 mM Na_2HPO_4 ('saline'). Fluorescence of the cellular suspension was monitored in thermostatted cuvettes (37°C) in a Perkin-Elmer MFP3 spectrofluorimeter (excitation 339 nm, emission 492 nm). Cells were stirred continuously except when tracings were interrupted for addition of Ca^{2+} or Mg^{2+}. Calibration of fluorescence signals was carried out as described in [8] by measuring fluorescence (F) after releasing intracellular dye with Triton X-100 (0.06–0.12%, v/v) in ≥1 mM Ca^{2+} (F_{max}) and again after addition of 10 mM EGTA and sufficient 1 M Tris base to achieve pH >8.3 (free Ca^{2+} < 1 nM, F_{min}). Cytosolic Ca^{2+} was calculated from the equation: $Ca^{2+} = 115\ nM\ (F - F_{min})/(F_{max} - F)$ [8]. Correction for changes in the autofluorescence of unloaded cells with the same additives was determined in each experiment. We showed previously that QUIN-2 has no effect on PTH release, Trypan blue exclusion, or cellular levels of K^+ and ATP in bovine parathyroid cells [1].

QUIN-2 was obtained from Lancaster Synthesis (Lancaster, England). TPA and the 4α- and 4β-isomers of phorbol 12,13-didecanoate were purchased from Sigma (St. Louis, MO). QUIN-2 and the phorbol esters were stored at −20°C in anhydrous dimethylsulfoxide. Other reagents were from sources cited before [6].

3. RESULTS

3.1. *Effects of TPA on PTH release*

In the absence of TPA, high extracellular Ca^{2+} concentrations caused a 60–70% inhibition of PTH release (fig.1A). TPA (10^{-6} M) reversed the inhibition of PTH secretion by high Ca^{2+} concentrations but had relatively little effect on hormonal release maximally stimulated by low extracellular

Ca^{2+} concentrations (fig.1A). This effect of the phorbol ester occurred rapidly and was persistent for at least 1 h (fig.1B). (In additional experiments TPA stimulated PTH release at 2 mM Ca^{2+} at 5 min. At this time the phorbol ester also stimulated PTH release at 0.5 mM Ca^{2+} by 1.6-fold.) Half of the maximal stimulation of PTH release by TPA at 2 mM Ca^{2+} occurred at 10 nM (fig.2). 4β-phorbol 12,13-didecanoate, which also activates protein kinase C [9], stimulated PTH release at 2 mM Ca^{2+} to an extent comparable to that observed with TPA (2 mM Ca^{2+} alone, 1.0 ± 0.05 ng/10^5 cells per h; 2 mM Ca^{2+} + 10^{-6} M TPA, 2.2 ± 0.1 ng/10^5 cells per h; 2 mM Ca^{2+} + 3×10^{-6} M 4β-phorbol 12,13-didecanoate, 2.3 ± 0.1 ng/10^5 cells per h). 4α-phorbol 12,13-didecanoate, however, which does not activate kinase C [9] had no effect on PTH release at concentrations as high as 10^{-5} M (2 mM Ca^{2+} + 10^{-5} M 4α-phorbol 12,13-didecanoate, 0.94 ± 0.07 ng/10^5 cells per h).

3.2. *Effect of TPA on cellular cAMP content and cytosolic Ca^{2+}*

TPA might stimulate PTH secretion at high Ca^{2+} concentrations indirectly, through changes in cellular cAMP [6] or cytosolic Ca^{2+} [1]. There was no effect of TPA (10^{-6} M), however, on cellular cAMP levels at either low (0.5 mM) or high (2 mM) extracellular Ca^{2+} concentrations (0.5 mM Ca^{2+}, 57 ± 4.1 fmol/10^5 cells; 0.5 mM Ca^{2+} + TPA, 54 ± 3.4 fmol/10^5 cells; 2 mM Ca^{2+}, 45.6 ± 1.7 fmol/10^5 cells; 2 mM Ca^{2+} + TPA, 42.8 ± 2.4 fmol/10^5 cells; 10 min incubation). Addition of TPA to QUIN-2-loaded parathyroid cells incubated with 1.5 mM Ca^{2+} likewise had no effect on cellular fluorescence (fig.3). In 5 experiments in which TPA (10^{-6} M) was added to cells prior to or after increasing extracellular Ca^{2+} from 0.5 to 1.5 mM, the cytosolic Ca^{2+} at 1.5 mM Ca^{2+} was equivalent in cells with or without TPA (508 ± 26 *vs* 522 ± 23 nM, respectively). TPA had no effect on the fluorescence of unloaded parathyroid cells.

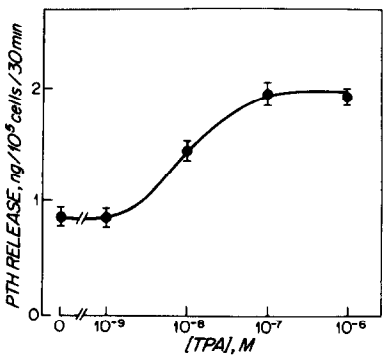

Fig.2. Dose-response for effect of TPA on PTH release. Cells were incubated for 30 min at 37°C with 2 mM Ca^{2+} and the indicated concentrations of TPA. Points represent the mean ± SEM for 6 observations in 2 experiments.

4. DISCUSSION

Unlike many other secretory cells, the parathyroid cell shows an inverse relationship between PTH release and the cytosolic Ca^{2+} concentration [1]. The basis for this 'backwards' secretory response to Ca^{2+} has not been established and might represent a unique exocytotic mechanism in this cell type. As in several other secretory cells, however, the phorbol ester TPA stimulates hormonal release in the parathyroid cell. Under conditions of reduced hormonal secretion (e.g., at high extracellular Ca^{2+} concentrations), TPA stimulates PTH release to levels comparable to those seen at low extracellular Ca^{2+} con-

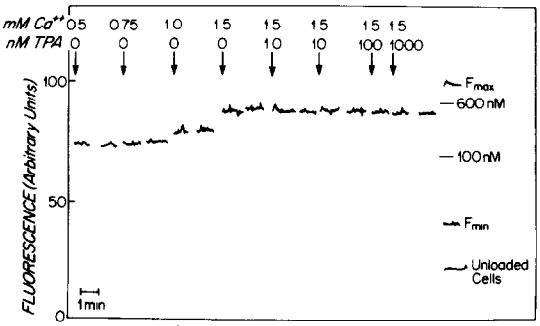

Fig.3. Effect of TPA on cellular fluorescence and cytosolic Ca^{2+} in QUIN-2-loaded parathyroid cells. Cells (10×10^6/ml) were loaded with QUIN-2 as described in section 2. Cellular fluorescence was then monitored (excitation 339 nm, emission 492 nm) at the indicated concentrations of Ca^{2+} and TPA. At the end of the experiment cells were lysed and calibration of the fluorescent signals was carried out as described in section 2. Cytosolic Ca^{2+} concentrations of 100 and 600 nm are shown, as is the autofluorescence of unloaded cells.

centrations. When secretion is already stimulated by low Ca^{2+}, on the other hand, TPA has relatively little additional effect.

The stimulation of PTH release by TPA at high extracellular Ca^{2+} concentrations is not associated with a reduction in cytosolic Ca^{2+}, unlike the effects of low Ca^{2+} concentrations. It is unlikely, therefore, that phorbol esters produce their effects on PTH secretion via changes in cytosolic Ca^{2+}. TPA also has no effect in cellular cAMP. Thus, phorbol esters do not appear to modulate PTH secretion by cAMP-dependent pathways.

The effects of TPA on exocytosis in platelets appear to be mediated by activation of protein kinase C [4]. Although we have no direct evidence that TPA activates this enzyme in parathyroid cells, several lines of evidence suggest that TPA stimulates PTH release via kinase C. First, the effects on PTH secretion are only observed with phorbol esters which activate protein kinase C (e.g., TPA and 4β-phorbol 12,13-didecanoate) and not with the inactive phorbol ester 4α-phorbol 12,13-didecanoate. Second, in preliminary studies [10], authors have found that high extracellular Ca^{2+} reduces the phosphorylation of several proteins in parathyroid cells and that addition of TPA at high Ca^{2+} restores the degree of phosphorylation to the level seen with low Ca^{2+}.

The data presented above suggest that stimulation of PTH release by low extracellular Ca^{2+} may be due to a low Ca^{2+}-induced activation of protein kinase C. Thus, the parathyroid cell may show a positive relationship between kinase C activity and hormonal secretion similar to that in other cells [4,11] and not have unique exocytotic mechanisms. It may appear paradoxical that protein kinase C activity would increase in the parathyroid cell at low extracellular Ca^{2+} concentrations when cytosolic Ca^{2+} is reduced, since the enzyme requires Ca^{2+} for activity [4]. In the presence of phospholipid and diacylglycerol, however, kinase C is active even at cytosolic Ca^{2+} concentrations present in the parathyroid cell at low extracellular Ca^{2+} concentrations ($\cong$200 nM) [4]. Changes in the level of diacylglycerol, therefore, would be a potential mediator for Ca^{2+}-regulated control of kinase C in the parathyroid. It is of interest that elevations in extracellular Ca^{2+} modify phospholipid turnover in the parathyroid cell [12] and, potentially, diacylglycerol availability. Further confirmation of this possibility will require direct determination of cellular levels of diacylglycerol in the parathyroid as a function of extracellular Ca^{2+} and their relationship to protein kinase C activity.

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