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PROTEIN KINASE C MODULATES HORMONE SECRETION REGULATED BY EXTRACELLULAR POLYCATIONS IN BOVINE PARATHYROID CELLS

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SUMMARY

1. The role of protein kinase C (PKC) in the regulation of parathyroid hormone (PTH) secretion was examined in dissociated bovine parathyroid cells.

2. Increasing the concentration of extracellular Ca^{2+} from 0·5 to 2 mM inhibited PTH secretion by 60–80%. Similar depressive effects on secretion were obtained by increasing the concentration of extracellular Mg^{2+} from 1 to 7 mM or by adding La^{3+} (to 40 μM). The PKC activator phorbol myristate acetate (PMA) depressed PTH secretion at the lower and potentiated secretion at the higher concentrations of extracellular Ca^{2+}, Mg^{2+} or La^{3+}. The inhibitory effect of PKC on secretion correlated positively with the magnitude of the inhibitory effect elicited by elevated extracellular Ca^{2+}.

3. The stimulatory effects of PKC activators on PTH secretion were reversed completely and the inhibitory effects were reversed partially by the PKC inhibitor staurosporine. Staurosporine alone did not affect secretion at low (0·5 mM) or high (2 mM) concentrations of extracellular Ca^{2+} but it did depress secretion at intermediate concentrations (around 1 mM) of extracellular Ca^{2+}.

4. The stimulatory effects of PKC activators on secretion were overcome by increases in the concentration of extracellular Ca^{2+} (to 5 or 10 mM) or La^{3+} (to 100 μM). In contrast, increasing the concentration of extracellular Mg^{2+} to 11 or 19 mM did not alleviate the potentiating effects of PKC activators. The different results obtained with Ca^{2+} and Mg^{2+} could not be explained by their different effects on cytosolic Ca^{2+} and suggests that different cations can have varying degrees of efficacy to activate functional responses linked to the Ca^{2+} receptor on bovine parathyroid cells.

5. PTH secretion stimulated by isoprenaline was not affected by PKC activators or staurosporine. Similarly, the inhibitory effects of extracellular ATPγS on secretion were unaffected by PKC activators. These results show that PKC activators affect specifically PTH secretion regulated by extracellular polycations.

6. The stimulatory effect of PKC activators on secretion parallels its inhibitory effects on $[Ca^{2+}]_i$ and inositol trisphosphate formation, showing that PKC blunts the

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mechanisms associated with extracellular Ca^{2+}-induced inhibition of secretion. The specificity of these actions suggests that PKC acts at a very early step of stimulus–secretion coupling in parathyroid cells, specific to that used by extracellular polycations and perhaps involving the Ca^{2+} receptor.

INTRODUCTION

The essential mechanism controlling bodily Ca^{2+} homeostasis centres on the parathyroid gland and its endocrine product, parathyroid hormone (PTH), the secretion of which is regulated by small changes in the concentration of extracellular Ca^{2+}. Elevated levels of extracellular Ca^{2+} depress secretion of PTH and cause corresponding increases in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in dissociated bovine (Shoback, Thatcher, Leombruno & Brown, 1984; Nemeth, Wallace & Scarpa, 1986) and human parathyroid cells (LeBoff *et al.* 1985; Larsson *et al.* 1984). This inverse relationship between $[Ca^{2+}]_i$ and secretion sets the parathyroid cell apart from most other secretory cells in which exocytotic secretion is typically coupled to or activated by increases in $[Ca^{2+}]_i$ (Douglas, 1974; Rubin, 1982; Cockcroft & Gomperts, 1988). In the parathyroid cell, the role of cytosolic Ca^{2+} in the regulation of secretion is controversial and while most data favour an inhibitory role (Shoback *et al.* 1984; Wallfelt *et al.* 1988) some data suggest that cytosolic Ca^{2+} can have both stimulatory and inhibitory effects on secretion (Nygren *et al.* 1987).

Studies on the regulation of cytosolic Ca^{2+} reveal that an increase in the concentration of extracellular Ca^{2+} causes two mechanistically distinct events: the mobilization of intracellular Ca^{2+} from a non-mitochondrial pool and the influx of extracellular Ca^{2+} through voltage-insensitive channels (Nemeth & Scarpa, 1986, 1987*a*; Muff, Nemeth, Haller-Brem & Fischer, 1988). These two mechanisms are reflected as transient and sustained, steady-state increases in $[Ca^{2+}]_i$, respectively. Various other di- and trivalent cations also cause the mobilization of intracellular Ca^{2+} (Nemeth, 1987). In contrast to extracellular Ca^{2+}, however, some of these other di- and trivalent cations (such as Mg^{2+} and La^{3+}) do not produce steady-state increases in $[Ca^{2+}]_i$. Yet all these cations depress PTH secretion similarly to extracellular Ca^{2+} (Nemeth, 1990). Moreover, lowering $[Ca^{2+}]_i$ by selectively blocking influx of extracellular Ca^{2+} does not augment PTH secretion (Nemeth & Scarpa, 1987*b*). Together, these findings demonstrate that sustained, steady-state increases in $[Ca^{2+}]_i$ resulting from the influx of extracellular Ca^{2+} are not involved in the short-term regulation of PTH secretion. It follows that the mobilization of intracellular Ca^{2+}, or some other receptor-dependent event associated with it, is more critically involved in the regulation of PTH secretion.

Although it is appealing to suppose that a receptor-mediated transient increase in $[Ca^{2+}]_i$ is sufficient to suppress PTH secretion, there is evidence that additional or alternative mechanisms may be involved (Nemeth *et al.* 1986; Nemeth & Scarpa, 1986, 1987*b*; Brown *et al.* 1987; Nemeth & Kosz, 1989). An attractive candidate is protein kinase C (PKC) which is activated by the receptor-dependent formation of diacylglycerol and which has been postulated to play a role in stimulus–secretion coupling in a variety of cells (Knight, 1986). In parathyroid cells, activators of PKC such as phorbol myristate acetate (PMA), have consistently been shown to augment

PTH secretion at high levels of extracellular Ca^{2+} (Brown, Redgrave & Thatcher, 1984; Muff & Fischer, 1986; Nemeth *et al.* 1986; Kobayashi, Russell, Lettieri & Sherwood, 1988; Morrissey, 1988; Muff & Fischer, 1989). In some studies, it has been reported that PMA also depresses PTH secretion at low levels of extracellular Ca^{2+} (Saxe, 1987; Membreno, Chen, Woodley, Gagucas & Shoback, 1989; Tanguay, Mortimer, Wood & Hanley, 1991). Increasing the concentration of extracellular Ca^{2+} increases the formation of diacyglycerol in parathyroid cells (Kifor & Brown, 1988) so it is likely that PKC activity changes as a function of the concentration of extracellular Ca^{2+}. This, in turn, suggests that PKC may play a physiological role in the regulation of PTH secretion.

In the present study we have examined the effects of various activators of PKC on PTH secretion regulated by extracellular polycations or the β-adrenergic or P_2 purinergic receptor. To assess the physiological significance of PKC, the effects of the PKC inhibitor staurosporine were examined. The results compliment the findings obtained in the previous paper which detail the actions of PKC on the regulation of $[Ca^{2+}]_i$ in parathyroid cells (Racke & Nemeth, 1993). Portions of the present study have appeared previously in abstract form (Nemeth, 1988; Racke & Nemeth, 1989).

METHODS

Buffers

Parathyroid cell buffer (PCB) contained (mM): NaCl, 126; KCl, 4; $MgSO_4$, 1; K_2HPO_4/KH_2HPO_4, 0·7; Na-Hepes, 20, pH 7·45, and variable amounts of $CaCl_2$ as indicated. PCB was usually supplemented with 1 mg/ml glucose and bovine serum albumin (BSA) (Miles Laboratories, Inc., West Haven, CT, USA; Fraction V) as specified. Percoll buffer was prepared as described previously (Nemeth & Scarpa, 1987*a*) except the final concentration of $CaCl_2$ was 1 mM. Measurements of PTH secretion were performed in PCB lacking phosphate and sulphate and containing 1 mM $MgCl_2$.

Preparation of dissociated bovine parathyroid cells

Parathyroid glands were obtained from freshly slaughtered calves (4–6 months old) at a local abattoir. Dissociated cells were prepared by collagenase digestion of minced tissue and were purified on continuous gradients of Percoll (Nemeth & Scarpa, 1987*a*; Nemeth & Kosz, 1989). Cells were subsequently washed several times in PCB containing 1·25 mM $CaCl_2$ and 0·5% BSA and then washed extensively under sterile conditions in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (F-12–DMEM) supplemented with 0·5% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml gentamicin. The cells were finally resuspended in F-12–DMEM supplemented with lower concentrations of antibiotics (10 U/ml penicillin, 10 μg/ml streptomycin, and 4 μg/ml gentamicin). This latter medium lacked serum and contained instead ITS^+ (insulin, transferrin, selenous acid, BSA, and linoleic acid). Cells in F-12–DMEM–ITS^+ were incubated for 16–24 h at 37 °C in a humid atmosphere of 5% CO_2 in air. The concentrations of antibiotics used for overnight culture are well below those which increase $[Ca^{2+}]_i$ and depress PTH secretion (see Racke & Nemeth, 1993). In the absence of serum, parathyroid cells did not attach to the flask and were collected for use in experiments by simply decanting the flasks after 12–24 h in primary culture.

Measurement of hormone secretion

Following overnight culture, the cells were removed from the flasks by decanting and washed with PCB–0·5 mM $CaCl_2$–0·1% BSA. The cells were resuspended in this same buffer and portions (0·2 ml) added to prewarmed plastic tubes (Falcon 2053) containing (or not) various test substances and/or varying concentrations of di- or trivalent cations. Incubations at 37 °C were typically for 30 min and were terminated by placing the tubes on ice. Cells were pelleted at 2 °C and 0·1 ml of the supernatant was stored at −70 °C.

The amount of PTH in supernatants was determined by radioimmunoassay using GW-1 antibody (generously provided by Dr Edward Brown) and an iodinated C-terminal fragment of human PTH as tracer ([^{125}I]PTH (65–84); INCSTAR, Stillwater, MN, USA). The GW-1 antibody

recognizes both intact and C-terminal fragments of PTH (Brown & Thatcher, 1982). Both bound and free fractions were counted and data reduction was performed using a logit transformation program. Duplicate samples from incubations performed in triplicate were assayed.

Cell concentrations were determined by counting nuclei. A portion of the cell suspension was diluted 10- or 20-fold with a solution containing 50 mM citrate, 0·05% cresyl violet and 0·02% Nonidet P40 (Sigma). After vigorous mixing, the nuclei, stained with cresyl violet, were counted in a haemocytometer.

Measurement of cytosolic Ca^{2+}

Parathyroid cells were cultured overnight as described above and loaded with fura-2 as described in an accompanying report (Racke & Nemeth, 1993). Fluorescent traces were calibrated after corrections for leakage of fura-2.

RESULTS

Stimulatory and inhibitory effects of PKC activators on PTH secretion

PTH secretion from parathyroid cells cultured overnight typically showed better suppressibility by extracellular divalent cations than cells used immediately after dissociation. The resting level of $[Ca^{2+}]_i$ was also lower in parathyroid cells following primary culture as described in Methods (Racke & Nemeth, 1993). For these reasons we have used primary cultures of parathyroid cells for the studies described herein.

Table 1 shows the stimulatory and inhibitory effects of PMA on PTH secretion. At a low concentration of extracellular Ca^{2+} (0·5 mM) PMA inhibited, whereas at a high concentration (2 mM) it potentiated PTH secretion. The effects of PMA on secretion were thus dependent on the concentration of extracellular Ca^{2+} and could be seen more clearly in concentration–response curves. The net effect of PMA was to flatten the concentration–response curve to extracellular Ca^{2+} (Fig. 1*A*). The potentiating effect of PMA on secretion was concentration dependent with an approximate EC_{50} of 10 nM and with maximal effects obtained at 100 nM (Fig. 1*B*). A nearly identical concentration–response curve is obtained for the inhibitory effects of PMA on the mobilization of intracellular Ca^{2+} evoked by extracellular divalent cations (Racke & Nemeth, 1993). The phorbol ester 4β-phorbol dibutyrate (PDBu), which activates PKC, behaved similarly to PMA although it was slightly less potent (the EC_{50} for potentiation of secretion at 2 mM extracellular Ca^{2+} was 30 nM). Phorbol esters that activate PKC thus blunt the inhibitory effects of extracellular Ca^{2+} on PTH secretion.

Other, structurally diverse activators of PKC had effects on PTH secretion similar to the active phorbol esters (Table 1). The potentiating effects of mezerein and (−)indolactam V on PTH secretion were concentration dependent (data not shown) and the concentrations used in Table 1 were those causing the greatest augmentation of secretion at 2 mM extracellular Ca^{2+}. The rank order of potency for augmenting PTH secretion was PMA > 4β-phorbol dibutyrate > (−)indolactam V > mezerein. This is the same rank order of potency of these compounds for activating PKC and for depressing the mobilization of intracellular Ca^{2+} evoked by extracellular divalent cations (Racke & Nemeth, 1993).

The ability of phorbol esters and (−)indolactam V to activate PKC is stereospecific, and the isomers of these compounds, when used at comparable concentrations, fail to influence PKC activity. The 4α-phorbols, even when used at concentrations 10-fold higher than active enantiomers, were without effect on secretion at low or high concentrations of extracellular Ca^{2+} (data not shown).

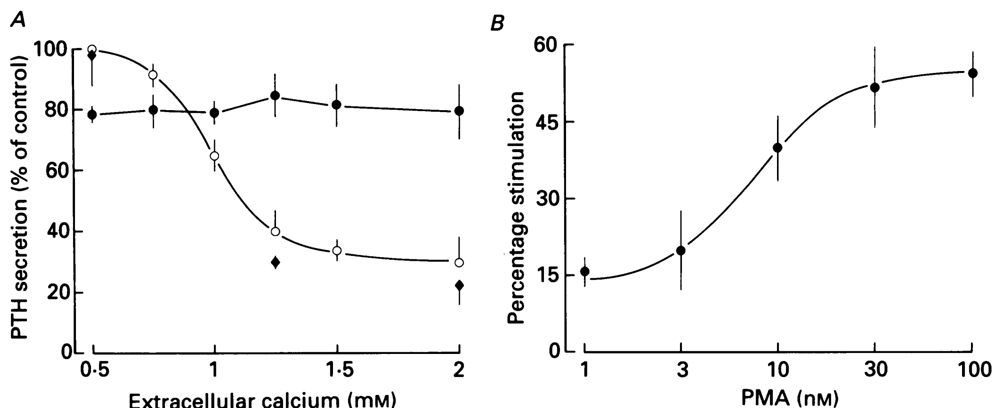

Fig. 1. Simulatory and inhibitory effects of PMA on PTH secretion. *A*, purified bovine parathyroid cells were incubated in the presence (●) or absence (○) of 100 nM PMA or 1 μM 4α-phorbol (◆) for 30 min at 37 °C with the indicated concentrations of extracellular Ca^{2+}. The results are expressed as a percentage of the control secretory response obtained in 0·5 mM extracellular Ca^{2+} which ranged from 4 to 30 ng/10^6 cells and averaged 14·0 ± 4·8 ng/10^6 cells ($n = 5$). Each point is the mean ± S.E.M. of three–five experiments. *B*, concentration-dependent stimulatory effects of PMA on PTH secretion, where 'percentage stimulation' is the percentage increase in secretion over control. Cells bathed in 2 mM extracellular Ca^{2+} were incubated for 30 min in the presence of the indicated concentration of PMA. Each point is the mean ± S.E.M. of three separate experiments.

TABLE 1. Stimulatory and inhibitory effects of PKC activators on PTH secretion and their reversal by staurosporine

	PTH secretion (ng/10^6 cells)			
			+ Staurosporine	
Treatment	0·5 mM $CaCl_2$	2 mM $CaCl_2$	0·5 mM $CaCl_2$	2 mM $CaCl_2$
None (control)	16·1 ± 1·6	3·6 ± 0·4	19·5 ± 1·9	4·0 ± 0·4
PMA (100 nM)	11·3 ± 1·3	11·3 ± 1·9	15·9 ± 0·9	3·7 ± 1·0
Mezerein (1 μM)	9·1 ± 1·0	8·8 ± 0·5	10·5 ± 1·3	3·9 ± 0·1
(−)Indolactam V (300 nM)	8·4 ± 0·7	7·2 ± 0·2	11·4 ± 1·5	3·6 ± 0·4

Parathyroid cells were incubated in buffer containing 0·5 or 2 mM $CaCl_2$ for 30 min in the presence or absence of the indicated concentration of PKC activator. In half the experiments, staurosporine (100 nM) was included during the incubation. Each value is the mean ± S.E.M. of twelve (control) or three (PKC activators) different experiments. The effects of all PKC activators on secretion in the absence of staurosporine were significantly different from control values ($P < 0·005$). In the presence of staurosporine, there was no significant difference between control and PKC activator-treated cells at 2 mM $CaCl_2$ although the inhibitory effects, obtained at 0·5 mM $CaCl_2$, were still significantly different from control values ($P < 0·05$).

(+)indolactam V (3 μM) similarly failed to augment secretion. The results obtained with these isomers suggest that the effects of PMA, PDBu, mezerein, and (−)indolactam V on PTH secretion result from an action on PKC.

As noted in the Introduction, various other di- and trivalent cations mimic the actions of extracellular Ca^{2+} and cause the mobilization of intracellular Ca^{2+} and inhibition of PTH secretion. Thus, increasing the concentration of extracellular Mg^{2+} from 1 to 7 mM inhibited PTH secretion by 60 ± 2 % ($n = 9$). Similarly, the addition of 40 μM La^{3+} caused a 61 ± 3 % ($n = 3$) inhibition of secretion. The inhibitory effects

of both Mg^{2+} and La^{3+} were blunted by PMA. The addition of 100 nM PMA significantly potentiated the secretory responses obtained at 7 mM Mg^{2+} and 40 μM La^{3+} by 39 ± 3 and 60 ± 20% ($P < 0.05$ for both), respectively. The effects of PMA therefore generalize the secretion regulated by a variety of di- and trivalent cations.

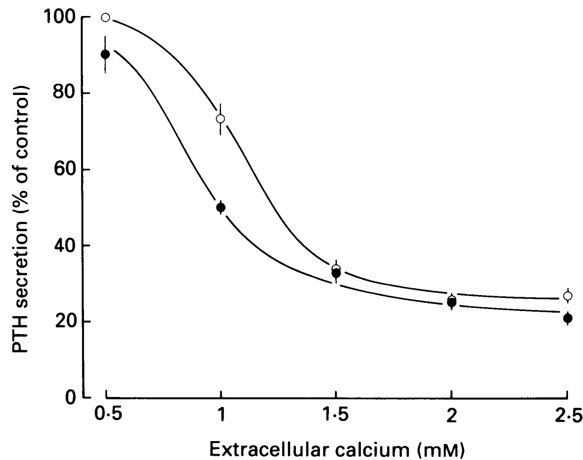

Fig. 2. Inhibitory effects of staurosporine on PTH secretion. Cells were incubated for 30 min with the indicated concentration of extracellular Ca^{2+} in the presence (●) or absence (○) of 100 nM staurosporine. Results are expressed as a percentage of the control secretory response obtained in 0·5 mM extracellular Ca^{2+} which ranged from 6·1 to 15·7 ng/10^6 cells and averaged 14·9 ng/10^6 cells. Each point is the mean ± S.E.M. of three experiments.

Effects of staurosporine on PTH secretion

Staurosporine was used at a concentration that maximally inhibits PKC activity and that reverses that depressive effects of PKC activators on intracellular Ca^{2+} mobilization in parathyroid cells (Racke & Nemeth, 1993). Staurosporine reversed completely the stimulatory effects of PKC activators on PTH secretion (Table 1). The inhibitory effects of these compounds, manifest at low concentrations of extracellular Ca^{2+}, were partially reversed by staurosporine.

The effect on PTH secretion of staurosporine alone was additionally examined in order to assess the physiological significance of PKC in stimulus–secretion coupling. By itself, staurosporine did not affect secretion at either low or high concentrations of extracellular Ca^{2+} (Fig. 2). However, staurosporine did cause a consistent depression of PTH secretion at intermediate (1 mM) concentrations of extracellular Ca^{2+} (27 ± 4% inhibition, $P < 0.005$; Fig. 2).

Supramaximal concentrations of extracellular polycations overcome the potentiating effects of PKC activators on PTH secretion

We noted previously that the depressive effects of PKC activators on the mobilization of intracellular Ca^{2+} could be overcome by increased concentrations of extracellular polycations (Nemeth, 1988; Racke, Meister & Nemeth, 1990). These preliminary studies showed that PKC activators shifted the concentration–response

curves for extracellular polycations to the right without affecting the maximal increase in $[Ca^{2+}]_i$. We therefore tested for parallels between the effects of PMA on changes in $[Ca^{2+}]_i$ and on secretion regulated by extracellular polycations.

Increasing the concentration of extracellular Ca^{2+} to 5 or 10 mM overcame the potentiating effect of PMA on PTH secretion (Fig. 3*A*). The net effect of PMA on

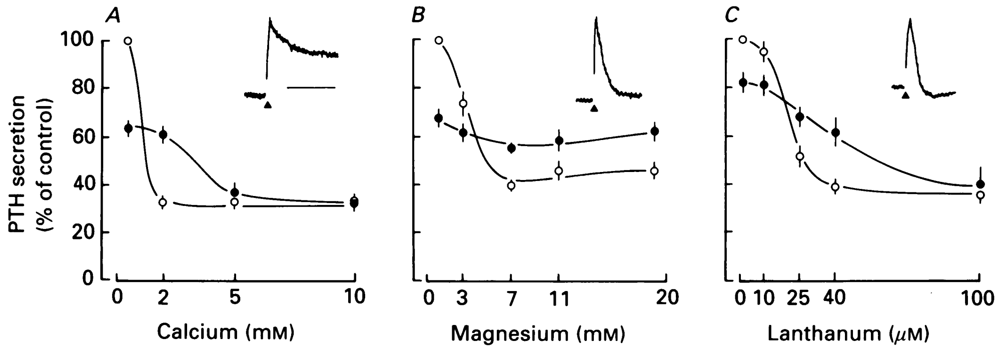

Fig. 3. Supramaximal concentrations of extracellular Ca^{2+} or La^{3+}, but not Mg^{2+}, overcome the potentiating effects of PMA on PTH secretion. Cells were incubated for 30 min with the indicated concentration of extracellular Ca^{2+} (*A*), Mg^{2+} (*B*), or La^{3+} (*C*) in the presence (●) or absence (○) of 100 nM PMA. In the experiments with Mg^{2+} and La^{3+}, the extracellular Ca^{2+} concentration was 0·5 mM. The results are expressed as a percentage of the respective control secretory responses which ranged from 3 to 17 ng/10^6 cells and averaged $7·6 \pm 1·3$, $12·7 \pm 1·8$, and $7·3 \pm 3·0$ ng/10^6 cells for the experiments with Ca^{2+}, Mg^{2+}, and La^{3+}, respectively. Each point is the mean ± S.E.M. of four (Ca^{2+}) or three (Mg^{2+} and La^{3+}) separate experiments. The control and PMA-treated values at 11 and 19 mM extracellular Mg^{2+} are significantly different ($P < 0·05$, paired *t* test). Insets: in some experiments, a portion of the cells were loaded with fura-2 and resuspended in buffer containing 0·5 mM extracellular Ca^{2+}. At the arrowheads, extracellular Ca^{2+}, Mg^{2+} or La^{3+} was added to a final concentration of 5 mM, 11 mM or 40 μM. The bar in *A* inset represents 1 min.

secretion thus paralleled its effect on the mobilization of intracellular Ca^{2+} evoked by extracellular Ca^{2+}; in both cases there was a shift to the right in the concentration–response curve that was unaccompanied by any change in the maximal response. The effects on secretion of mezerein or (−)indolactam V were also reversed by elevated levels of extracellular Ca^{2+}. In three experiments, increasing the concentration of extracellular Ca^{2+} from 0·5 to 5 mM depressed PTH secretion from $10·77 \pm 1·88$ to $2·16 \pm 0·30$ ng/10^6 cells. This degree of inhibition was the same in the presence of 1 μM mezerein ($2·18 \pm 0·04$) or 300 nM (−)indolactam V ($2·16 \pm 0·42$). These results agree well with those obtained by Membreno *et al.* (1989) who showed that the potentiating effects of some other activators of PKC (bryostatin I and dioctanoylglycerol) were similarly overcome by increases in the concentration of extracellular Ca^{2+}.

In contrast to extracellular Ca^{2+}, however, increasing the concentration of extracellular Mg^{2+} to 11 or 19 mM failed to overcome the potentiating effect of PKC activators on secretion. This result, shown for PMA in Fig. 3*B*, was rather surprising, since a variety of other studies have shown a remarkable degree of similarity between the actions of these two divalent cations on parathyroid cells (Nemeth, 1990; Brown,

1991). One notable exception, however, is the differential effects of extracellular Ca^{2+} and Mg^{2+} on $[Ca^{2+}]_i$. Although both cations evoke transient increases in $[Ca^{2+}]_i$, only extracellular Ca^{2+} causes sustained, steady-state increases in $[Ca^{2+}]_i$ (Fig. 3, insets). In bovine parathyroid cells, extracellular Mg^{2+} does not cause influx of extracellular Ca^{2+} and perhaps it is the sustained increase in $[Ca^{2+}]_i$, resulting from influx, that enables extracellular Ca^{2+}, but not Mg^{2+}, to overcome the potentiating effects of PMA on secretion.

This hypothesis was tested by examining the ability of supramaximal concentrations of La^{3+} to overcome the potentiating effects of PMA on secretion. Concentrations of La^{3+} that depress PTH secretion cause transient increases in $[Ca^{2+}]_i$ that arise from the mobilization of intracellular Ca^{2+} (Nemeth, 1987). But because La^{3+} is an effective blocker of Ca^{2+} influx in parathyroid cells (Nemeth & Scarpa, 1986, 1987*a*) and in cells generally (dos Remedios, 1981), the cytosolic Ca^{2+} transients are unaccompanied by sustained, steady-state increases in $[Ca^{2+}]_i$ and in this respect resemble the response to extracellular Mg^{2+} (Fig. 3, insets). We found, however, that supramaximal concentrations of La^{3+} overcame the potentiating effect of PMA on secretion (Fig. 3*C*). It follows that the ability of extracellular Ca^{2+} to reverse the potentiating effects of PMA on secretion is unrelated to sustained, steady-state increases in $[Ca^{2+}]_i$ resulting from the influx of extracellular Ca^{2+}.

Relationship between the inhibitory effects of extracellular Ca^{2+} and PKC activators on PTH secretion

During the course of these studies, we noted some variability in the inhibitory effects of PKC activators on secretion obtained at low (0·5 mM) concentrations of extracellular Ca^{2+}. In most experiments, PKC activators depressed secretion by anywhere from 20 to 70% but in a few experiments, potentiation was seen. These exceptional results were observed only in cell preparations that responded poorly to increases in extracellular Ca^{2+} and were therefore omitted in the description of the effects of PKC activators presented above. Quantitative evaluation of all experiments revealed that the magnitude of the inhibitory effect of PKC activators was related to the magnitude of inhibition produced by elevated levels of extracellular Ca^{2+}. This relationship is shown in Fig. 4 which summarizes a number of experiments using PMA, mezerein, or (−)indolactam V. The inhibitory effects of PKC activators at low concentrations of extracellular Ca^{2+} correlated significantly with the degree of suppression achieved by 2 mM extracellular Ca^{2+}. Thus, when extracellular Ca^{2+} caused a profound inhibition of secretion, then PKC activators also depressed secretion to a large degree. When extracellular Ca^{2+} caused a meagre depression of secretion, then PKC activators inhibited considerably less and, in some cases, potentiated secretion.

Effects of PKC activators and staurosporine on secretion evoked by isoprenaline or ATPγS

Secretion of PTH has long been known to be stimulated by β-adrenergic agonists or agents that elevate cyclic AMP (Brown & Aurbach, 1980). β-Adrenergic agonists such as isoprenaline potentiate PTH secretion at all concentrations of extracellular Ca^{2+} although extracellular Ca^{2+} is still capable of depressing secretion (Brown,

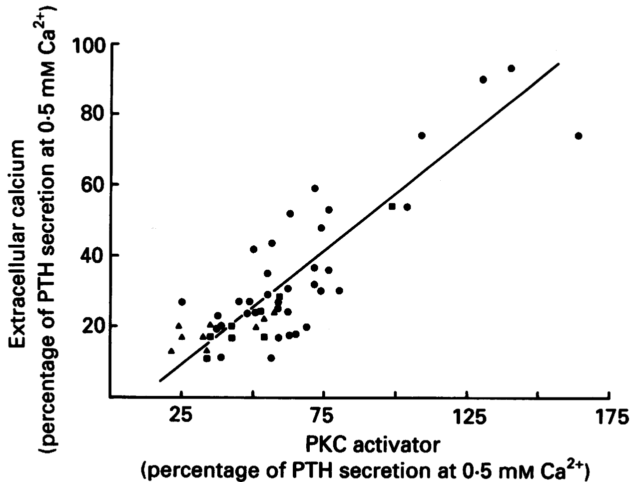

Fig. 4. Inhibitory effects of PKC on PTH secretion correlate with the degree of suppressibility by extracellular Ca^{2+}. Parathyroid cells were incubated with 0·5 mM extracellular Ca^{2+} for 30 min in the presence of 100 nM PMA (●), 300 nM (−)indolactam V (▲) or 1 μM mezerein (■). Other cells from the same batch were incubated with 2 mM extracellular Ca^{2+}. The results are expressed as a percentage of the secretory response obtained in the absence of PKC activators and 0·5 mM Ca^{2+}. The effects of PKC activators on PTH secretion in 0·5 mM $CaCl_2$ (abscissa) were plotted against the effects on secretion of 2 mM $CaCl_2$ (ordinate) in the same batch of cells. Each point is the mean of triplicate determinations using a different batch of cells. Linear regression analysis resulted in a line described by $y = -5{\cdot}408 + 0{\cdot}616x$ with $r^2 = 0{\cdot}763$ ($P < 0{\cdot}0001$ by analysis of variance).

TABLE 2. Secretory responses to isoprenaline are unaffected by PMA or mezerein

Treatment	PTH secretion (ng/10^6 cells) 0·5 mM $CaCl_2$	2 mM $CaCl_2$
None (control)	16·2 ± 2·3	4·3 ± 0·8
Isoprenaline (10 μM)	24·3 ± 1·8	6·3 ± 0·9
PMA (100 nM)	11·3 ± 1·1	10·0 ± 1·2
Mezerein (1 μM)	9·3 ± 1·2	8·5 ± 1·2
Isoprenaline + PMA	17·1 ± 2·0	19·0 ± 1·8
Isoprenaline + mezerein	13·7 ± 1·6	14·9 ± 1·2

Cells were incubated for 30 min in the presence or absence of the PKC activator with or without isoprenaline in buffer containing 0·5 or 2 mM $CaCl_2$. Each value is the mean ± S.E.M. of three experiments.

Gardner, Windeck & Aurbach, 1978). This is shown in Table 2. Isoprenaline potentiated secretion at low and at high concentrations of extracellular Ca^{2+} to similar extents (by 1·5- and 1·4-fold, respectively). However, the percentage inhibition of secretion caused by extracellular Ca^{2+} was the same in the absence (73 % inhibition) and presence (74 % inhibition) of isoprenaline. The PKC activators PMA and mezerein depressed isoprenaline-stimulated secretion at low concentrations of extracellular Ca^{2+} and potentiated it at high extracellular Ca^{2+}, in a manner similar to their effects in the absence of isoprenaline. Significantly, neither PMA nor

mezerein altered the potentiating effect of isoprenaline obtained at either 0·5 or 2 mM extracellular Ca^{2+}. Thus, at 0·5 mM extracellular Ca^{2+}, isoprenaline caused a 1·5-fold increase in PTH secretion in the presence of absence of PMA. At 2 mM extracellular Ca^{2+}, isoprenaline caused a 1·4- and 1·9-fold increase in PTH secretion in the absence and presence of PMA, respectively. Similar effects were obtained with mezerein.

The stimulatory effects of isoprenaline on PTH secretion were also unaffected by staurosporine (data not shown). The results obtained with staurosporine compliment those obtained with PKC activators and together show that the potentiating effects of isoprenaline on PTH secretion are not affected by activators or an inhibitor of PKC.

Parathyroid cells also possess a P_2 purinoceptor activated by extracellular ATP (Nemeth & Kosz, 1989). It is particularly relevant to study the effects of PKC activators on PTH secretion regulated by the P_2 receptor because extracellular ATP, like extracellular Ca^{2+}, causes the mobilization of intracellular Ca^{2+} and an inhibition of PTH secretion. ATPγS was used in these experiments because it is only slowly hydrolysed and acts as an agonist at the P_2 receptor on parathyroid cells. Incubation of parathyroid cells (bathed in 0·5 mM extracellular Ca^{2+}) for 30 min with 50 μM ATPγS inhibited PTH secretion by 67 ± 7 and by 65 ± 6 % ($n = 6$) in the absence and presence of PMA (100 nM), respectively. As in previous experiments, the addition of PMA alone depressed secretion by 51 ± 6 %, but it did not block the inhibitory effects of ATPγS. Thus, both stimulatory and inhibitory effects on PTH secretion mediated by other receptors are insensitive to activators or an inhibitor of PKC.

DISCUSSION

The results of the present study show that PKC activators have both inhibitory and stimulatory effects on PTH secretion that are dependent on the concentration of extracellular Ca^{2+}. The inhibitory effects of staurosporine are similarly dependent on the concentration of extracellular Ca^{2+}. Although the pharmacological specificity of both the inhibitory and stimulatory effects of PKC activators suggests that they result from an action on PKC, there are reasons for supposing that PKC affects different mechanisms to achieve these effects. While the mechanism(s) affected by PKC that result in inhibition of PTH secretion are unclear, the mechanism resulting in a stimulation of secretion seems more certain. And it is the stimulatory effect of PKC on secretion which appears to reveal a physiological role for PKC in stimulus–secretion coupling in parathyroid cells. The effects of PKC on PTH secretion are thus considered separately.

The inhibitory effect of PKC on secretion

Although the stimulatory effect of PKC activators on PTH secretion has been noted consistently in previous reports, the inhibitory effect, observed at low concentrations of extracellular Ca^{2+}, has been observed in only some of these studies. The present data show that the inhibitory effects of PKC activators correlate positively with those achieved by extracellular Ca^{2+}; PKC activators do not inhibit secretion when the inhibitory response to extracellular Ca^{2+} is small. These data offer one possible explanation for the discrepant observations observed previously.

Previous studies can be divided on the basis of the magnitude of suppression of PTH secretion effected by extracellular Ca^{2+}. Thus, when extracellular Ca^{2+} depressed PTH secretion by $< 50\%$, PKC did not inhibit secretion at low concentrations of extracellular Ca^{2+} (Muff & Fischer, 1986, 1989; Nemeth *et al.* 1986; Morrissey, 1988). In contrast, in those reports which achieved $> 50\%$ suppressibility by extracellular Ca^{2+}, an inhibitory effect of PKC activators on PTH secretion was observed (Brown *et al.* 1984; Saxe, 1987; Membreno *et al.* 1989; Tanguay *et al.* 1991).

It seems likely that the inhibitory and stimulatory effects of PKC activators on PTH secretion result from PKC affecting different mechanisms. Thus, the inhibitory effects are only partially reversed by staurosporine whereas the stimulatory effects are reversed completely. Moreover, the concentration–response curves for PMA effects on PTH secretion are slightly different: inhibitory effects are seen at lower concentrations of PMA than are the stimulatory effects (Saxe, 1987). Finally, it is possible to dissociate the inhibitory and stimulatory effects by prolonged incubation (24 h) with PMA (F. K. Racke & E. K. Nemeth, in preparation). Thus, a single mechanism is unlikely to explain both the inhibitory and stimulatory effects of PKC activators on PTH secretion.

At present, the site of action of PKC that could explain its inhibitory effect on PTH secretion is unknown. Activators of PKC do not cause any change in $[Ca^{2+}]_i$ under conditions (low extracellular Ca^{2+}) where they depress PTH secretion (Racke & Nemeth, 1993). PMA does, however, increase the formation of diacylglycerol when extracellular Ca^{2+} levels are low (Racke & Nemeth, 1993). Raising the concentration of extracellular Ca^{2+} similarly increases the formation of diacylglycerol (Kifor & Brown, 1988). Perhaps the increased formation of diacyglycerol caused by PMA at a low concentration of extracellular Ca^{2+} is somehow related to its inhibitory effect on PTH secretion.

The stimulatory effect of PKC on secretion

The stimulatory effect of PKC on PTH secretion compliments the accompanying report (Racke & Nemeth, 1993) and shows that the secretory response of the parathyroid cell to extracellular Ca^{2+} is affected by PKC similarly to $[Ca^{2+}]_i$. In both cases there is a shift to the right in the concentration–response curve for extracellular Ca^{2+} and the maximal response, whether it be an increase in $[Ca^{2+}]_i$ or a decrease in PTH secretion, can be obtained at higher concentrations of extracellular Ca^{2+}.

The finding that extracellular Mg^{2+} did not overcome the potentiating effects of PMA on secretion shows that it behaves somewhat differently from extracellular Ca^{2+}. In bovine parathyroid cells, Mg^{2+} does not promote the influx of extracellular Ca^{2+} (although it does so in human parathyroid cells; Nemeth, 1990) so there is already some evidence showing that these two divalent cations are not quite equivalent stimuli for bovine parathyroid cells. We suggest that Mg^{2+} is not only less potent, but additionally less efficacious in regulating bovine parathyroid cell functions. Presumably, extracellular Ca^{2+} activates some additional mechanism that is insensitive to extracellular Mg^{2+} and this enables Ca^{2+} to overcome the stimulant effects of PMA on PTH secretion.

In the prior paper (Racke & Nemeth, 1993), it was shown that the effect of PMA on $[Ca^{2+}]_i$ parallels its effect on inositol trisphosphate formation. The ability of PKC

to depress these physiological and biochemical parameters similarly shows that PKC is blocking the mechanisms associated with extracellular Ca^{2+}-induced inhibition of PTH secretion. The stimulatory effect of PKC on secretion is therefore more correctly described as disinhibition, since it depresses the mechanisms associated with an inhibition of PTH secretion.

These observations, together with the results obtained using staurosporine, suggest that PKC may act to depress these transmembrane and cytosolic signalling mechanisms under physiological conditions and thereby play a role in stimulus–secretion coupling in parathyroid cells. Thus, staurosporine augmented extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and depressed PTH secretion. Both these effects were most prominent at physiological levels of extracellular Ca^{2+}. We tend to place some emphasis on the effects of staurosporine, since an inhibitor can provide insights into the mechanisms normally used by extracellular Ca^{2+} and essential to the regulation of PTH secretion whereas activators can influence secretion through alternative routes. The finding that an inhibitor of PKC affects PTH secretion under normocalcaemic conditions suggests that PKC activation is normally involved in the modulation of secretion in parathyroid cells. Increased PKC activity may thus function in a negative feedback capacity to damp the signalling mechanisms used by extracellular Ca^{2+}. This single action can adequately explain the stimulatory effects of PKC on PTH secretion and there is no need to postulate any stimulatory effect of PKC on exocytosis directly.

Mechanism of action of PKC on PTH secretion

The finding that PTH secretion stimulated by isoprenaline or inhibited by ATPγS is unaffected by PKC activators or staurosporine suggests that PKC acts specifically on the mechanism used by extracellular polycations to regulate secretion. The failure to influence secretion regulated by ATPγS is most significant because the P_2 purinergic receptor, like the Ca^{2+} receptor, is coupled to the mobilization of intracellular Ca^{2+} and inhibition of PTH secretion. This suggests a site of action of PKC very early on in the process of stimulus–secretion coupling and used exclusively by extracellular polycations. An action of PKC on the Ca^{2+} receptor itself is a most attractive hypothesis because this single action could explain the specificity of the effects of PKC on both $[Ca^{2+}]_i$ and secretion. Moreover, there is a precedent for PKC-dependent phosphorylation of other Ca^{2+}-mobilizing receptors which results in depression of evoked increases in inositol trisphosphate formation and in $[Ca^{2+}]_i$ (Lebb-Lundberg, Cotecchia, Lomasney, DeBernardis, Lefkowitz & Caron, 1985). If PKC acts specifically on the Ca^{2+} receptor, then it would not affect $[Ca^{2+}]_i$ or secretion regulated by ATPγS; and this was the result obtained. But an action of PKC to depress Ca^{2+} receptor function would probably affect other mechanisms coupled to this receptor that may be essential in the regulation of PTH secretion and that do not involve changes in $[Ca^{2+}]_i$. Presumably, depression of this alternative, Ca^{2+}-independent mechanism is overcome by supramaximal concentrations of extracellular Ca^{2+}. Thus, an action of PKC on the Ca^{2+} receptor could explain its ability to impair both cytosolic Ca^{2+}-dependent and -independent mechanisms involved in the regulation of PTH secretion.

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REFERENCES

BROWN, E. M. (1991). Extracellular calcium sensing, regulation of parathyroid cell function and the role of calcium and other ions as extracellular (first) messengers. *Physiological Reviews* **71**, 371–411.

BROWN, E. M. & AURBACH, G. D. (1980). Role of cyclic nucleotides in secretory mechanisms and actions of parathyroid hormone and calcitonin. *Vitamins and Hormones* **38**, 205–256.

BROWN, E. M., GARDNER, D. G., WINDECK, R. A. & AURBACH, G. D. (1978). Relationship of intracellular 3′,5′-adenosine monophosphate accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology* **103**, 2323–2333.

BROWN, E., REDGRAVE, J. & THATCHER, J. (1984). Effect of the phorbol ester TPA on PTH secretion. *FEBS Letters* **175**, 72–75.

BROWN, E. M. & THATCHER, J. G. (1982). Adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase and the regulation of parathyroid hormone release by divalent cations and agents elevating cellular cAMP in dispersed bovine parathyroid cells. *Endocrinology* **110**, 1374–1380.

BROWN, E. M., WATSON, E. J., THATCHER, J. G., KOLETSKY, R., DAWSON-HUGHES, B. F., POSILLICO, J. T. & SHOBACK, D. M. (1987). Ouabain and low extracellular potassium inhibit PTH secretion from bovine parathyroid cells by a mechanism that does not involve increases in the cytosolic calcium concentration. *Metabolism* **36**, 36–42.

COCKCROFT, S. & GOMPERTS, B. D. (1988). Some new questions concerning the role of Ca^{2+} in exocytosis. In *Handbook of Experimental Pharmacology*, vol. 83, ed. BAKER, P. F., pp. 305–338. Springer-Verlag, New York.

DOS REMEDIOS, C. G. (1981). Lanthanide ion probes of calcium-binding sites on cellular membranes. *Cell Calcium* **2**, 29–51.

DOUGLAS, W. W. (1974). Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. *Biochemical Society Symposium* **39**, 1–28.

KIFOR, O. & BROWN, E. M. (1988). Relationship between diacylglycerol levels and extracellular Ca^{2+} in dispersed bovine parathyroid cells. *Endocrinology* **123**, 2723–2729.

KNIGHT, D. E. (1986). Calcium and exocytosis. In *Calcium and the Cell*, Ciba Foundation Symposium No. 122, ed. EVERED, D. & WHELAN, J., pp. 250–265. John Wiley & Sons, New York.

KOBAYASHI, N., RUSSELL, J., LETTIERI, D. & SHERWOOD, L. M. (1988). Regulation of protein kinase C by extracellular calcium in bovine parathyroid cells. *Proceedings of the National Academy of Sciences of the USA* **85**, 4857–4860.

LARSSON, R., WALLFELT, C., ABRAHAMSSON, H., GYLFE, E., LJUNGHALL, S., RASTAD, J., RORSMAN, P., WIDE, L. & AKERSTROM, G. (1984). Defective regulation of the cytosolic Ca^{2+} activity in parathyroid cells from patients with hyperparathyroidism. *Bioscience Reports* **4**, 909–915.

LEBB-LUNDBERG, L. M. F., COTECCHIA, S., LOMASNEY, J. W., DEBERNARDIS, J. F., LEFKOWITZ, R. J. & CARON, M. G. (1985). Phorbol esters promote $alpha_1$-adrenergic receptor uncoupling from inositol phospholipid metabolism. *Proceedings of the National Academy of Sciences of the USA* **82**, 5651–5655.

LEBOFF, M. S., SHOBACK, D., BROWN, E. M., THATCHER, J., LEOMBRUNO, R., BEAUDION, D., HENRY, M., WILSON, R., PALLOTTA, J., MARYNICK, S., STOCK, J. & LEIGHT, G. (1985). Regulation of parathyroid hormone release and cytosolic calcium by extracellular calcium in dispersed and cultured bovine and pathological human parathyroid cells. *Journal of Clinical Investigation* **75**, 49–57.

MEMBRENO, L., CHEN, T., WOODLEY, D., GAGUCAS, R. & SHOBACK, D. (1989). The effects of protein kinase C agonists on parathyroid hormone release and intracellular free Ca^{2+} in bovine parathyroid cells. *Endocrinology* **124**, 789–797.

MORRISSEY, J. J. (1988). Effect of phorbol myristate acetate on secretion of parathyroid hormone. *American Journal of Physiology* **254**, E63–70.

MUFF, R. & FISCHER, J. A. (1986). Stimulation of parathyroid hormone secretion by phorbol esters is associated with a decrease of cytosolic calcium. *FEBS Letters* **194**, 215–218.

MUFF, R. & FISCHER, J. A. (1989). Trypsinization of bovine parathyroid cells abolishes Ca^{2+}-regulated parathyroid hormone secretion. *Journal of Endocrinology* **122**, 213–218.

MUFF, R., NEMETH, E. F., HALLER-BREM, S. & FISCHER, J. A. (1988). Regulation of hormone secretion and cytosolic Ca^{2+} by extracellular Ca^{2+} in parathyroid cells and C-cells: Role of voltage-sensitive Ca^{2+} channels. *Archives of Biochemistry and Biophysics* **265**, 128–135.

NEMETH, E. F. (1987). Evidence for the presence of a novel Ca^{2+}-binding protein (Ca^{2+} receptor) on the surface of parathyroid cells. In *Calcium-Binding Proteins in Health and Disease*, ed. NORMAN, A. W., VANAMAN, T. C. & MEANS, A. R., pp. 36–38. Academic Press, New York.

NEMETH, E. F. (1988). Phorbol esters dissociate changes in cytosolic calcium from hormone secretion in parathyroid cells. *Journal of Bone and Mineral Research* **3**, S212.

NEMETH, E. F. (1990). Regulation of cytosolic calcium by extracellular divalent cations in C-cells and parathyroid cells. *Cell Calcium* **11**, 323–327.

NEMETH, E. F. & KOSZ, L. M. (1989). Adenine nucleotides mobilize cellular Ca^{2+} and inhibit parathyroid hormone secretion. *American Journal of Physiology* **257**, E505–513.

NEMETH, E. F. & SCARPA, A. (1986). Cytosolic Ca^{2+} and the regulation of secretion in parathyroid cells. *FEBS Letters* **203**, 15–19.

NEMETH, E. F. & SCARPA, A. (1987*a*). Rapid mobilization of cellular Ca^{2+} in bovine parathyroid cells evoked by extracellular divalent cations. Evidence for a cell surface calcium receptor. *Journal of Biological Chemistry* 262, 5188–5196.

NEMETH, E. F. & SCARPA, A. (1987*b*). Are changes in intracellular free calcium necessary for regulating secretion in parathyroid cells? *Annals of the New York Academy of Sciences* **493**, 542–551.

NEMETH, E. F., WALLACE, J. & SCARPA, A. (1986). Stimulus–secretion coupling in bovine parathyroid cells. Dissociation between secretion and net changes in cytosolic Ca^{2+}. *Journal of Biological Chemistry* **261**, 2668–2674.

NYGREN, P., LARSSON, R., LINDH, E., LJUNGHALL, S., RASTAD, J., AKERSTROM, G. & GYLFE, E. (1987). Bimodal regulation of secretion by cytoplasmic Ca^{2+} as demonstrated by the parathyroid. *FEBS Letters* **213**, 195–198.

RACKE, F. K., MEISTER, M. A. & NEMETH, E. F. (1990). Transmembrane signalling pathways activated by extracellular metal cations in parathyroid cells: differential effects of phorbol myristate acetate and pertussis toxin. *Journal of Bone and Mineral Research* **5**, S260.

RACKE, F. K. & NEMETH, E. F. (1989). Modulation of cytosolic calcium and hormone secretion by protein kinase C in parathyroid cells. *Journal of Bone and Mineral Research* **4**, S144.

RACKE, F. K. & NEMETH, E. F. (1993). Cytosolic calcium homeostasis in bovine parathyroid cells and its modulation by protein kinase C. *Journal of Physiology* **468**, 141–162.

RUBIN, R. P. (1982). *Calcium and Cellular Secretion*. Plenum Press, New York.

SAXE, A. W. (1987). The effect of phorbol ester on in vitro release of parathyroid hormone from abnormal human parathyroid cells. *Surgery* **102**, 932–940.

SHOBACK, D. M., THATCHER, J., LEOMBRUNO, R. & BROWN, E. M. (1984). Relationship between parathyroid hormone secretion and cytosolic calcium concentration in dispersed bovine parathyroid cells. *Proceedings of the National Academy of Sciences of the USA* **81**, 3113–3117.

TANGUAY, K. E., MORTIMER, S. T., WOOD, P. H. & HANLEY, D. A. (1991). The effects of phorbol myristate acetate on the intracellular degradation of bovine parathyroid hormone. *Endocrinology* **128**, 1863–1868.

WALLFELT, C., LINDH, E., LARSSON, R., JOHANSSON, H., RASTAD, J., AKERSTROM, G. & GYLFE, E. (1988). Kinetic evidence for cytoplasmic calcium as an inhibitory messenger in parathyroid hormone release. *Biochimica et Biophysica Acta* **969**, 257–262.