Cyclic AMP stimulates luteinizing-hormone (lutropin) exocytosis in permeabilized sheep anterior-pituitary cells

Synergism with protein kinase C and calcium

M. Bruce MACRAE, James S. DAVIDSON, Robert P. MILLAR and P. Anton van der MERWE*

Medical Research Council Regulatory Peptides Research Unit, Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa

Sheep anterior-pituitary cells permeabilized with *Staphylococcus aureus* α -toxin were used to investigate the role of cyclic AMP (cAMP) in exocytosis of luteinizing hormone (lutropin, LH) under conditions where the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{free}}$) is clamped by Ca^{2+} buffers. At resting $[\text{Ca}^{2+}]_{\text{free}}$ (pCa 7), cAMP rapidly stimulated LH exocytosis (within 5 min) and continued to stimulate exocytosis for at least 30 min. When cAMP breakdown was inhibited by 3-isobutyl-1-methylxanthine (IBMX), the concentration giving half-maximal response (EC_{50}) for cAMP-stimulated exocytosis was $10 \mu\text{M}$. cAMP-stimulated exocytosis required millimolar concentrations of MgATP, as has been found with Ca^{2+} - and phorbol-ester-stimulated LH exocytosis. cAMP caused a modest enhancement of Ca^{2+} -stimulated LH exocytosis by decreasing in the EC_{50} for Ca^{2+} from pCa 5.6 to pCa 5.9, but had little effect on the maximal LH response to Ca^{2+} . Activation of protein kinase C (PKC) with phorbol 12-myristate 13-acetate (PMA) dramatically enhanced cAMP-stimulated LH exocytosis by both increasing the maximal effect 5–7-fold and decreasing the EC_{50} for cAMP to $3 \mu\text{M}$. This synergism between cAMP and PMA was further augmented by increasing the $[\text{Ca}^{2+}]_{\text{free}}$. Gonadotropin-releasing hormone (gonadoliberin, GnRH) stimulated cAMP production in intact pituitary cells. Since GnRH stimulation is reported to activate PKC and increase the intracellular $[\text{Ca}^{2+}]_{\text{free}}$, our results suggest that a synergistic interaction of the cAMP, PKC and Ca^{2+} second-messenger systems is of importance in the mechanism of GnRH-stimulated LH exocytosis.

INTRODUCTION

Although it is well established that Ca^{2+} is a major second messenger mediating GnRH-stimulated LH exocytosis [1], the role of cAMP is controversial. Early reports claiming a second-messenger role for cAMP [2–6] were not confirmed in subsequent work [7–10]. Since these studies were performed in intact cells, the effects of cAMP on LH exocytosis were examined by using high concentrations of membrane-permeant cAMP analogues and by activating adenylate cyclase with forskolin [2–10]. These approaches have pitfalls, since cAMP analogues are used at concentrations at which they interact with the GnRH receptor [11,12], and forskolin is not entirely specific [13]. More importantly, studies in intact cells are complicated by interactions between the second-messenger systems. For example, cAMP can activate voltage-sensitive plasma-membrane Ca^{2+} channels [14–16] and stimulate an increase in intracellular $[\text{Ca}^{2+}]_{\text{free}}$ [17,18]. This could explain the finding that forskolin-stimulated LH secretion is dependent on extracellular Ca^{2+} and is inhibited by voltage-sensitive Ca^{2+} -channel blockers [19].

By utilizing permeabilized cells, cAMP can be introduced directly into the cell and the intracellular $[\text{Ca}^{2+}]_{\text{free}}$ can be clamped by using Ca^{2+} buffers [20]. We previously characterized Ca^{2+} - and phorbol-ester-stimulated LH exocytosis in sheep anterior-pituitary cells permeabilized with *Staphylococcus aureus* α -toxin [20]. In the present study the same methodology has been used to investigate (a) the ability of cAMP to stimulate LH exocytosis and (b) the effect of cAMP on Ca^{2+} - and phorbol-ester-stimulated

LH exocytosis. Our results demonstrate a synergism of cAMP with these stimulators of exocytosis and suggest an important role for cAMP as an intracellular mediator of acute LH exocytosis.

MATERIALS AND METHODS

Materials

Staph. aureus α -toxin was obtained from Dr. Sucharit Bhakdi (Institute of Medical Microbiology, Justus-Liebig University, Giessen, Germany). Ovine LH (NIADDK-oLH-I-3) and anti-serum to ovine LH (NIADDK-anti-oLH-1) were kindly provided by the NIDDK. Mammalian GnRH was synthesized by Dr R. C. deL. Milton, Department of Chemical Pathology, University of Cape Town. Other Chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Permeabilization and cell stimulation

Primary sheep anterior-pituitary cell cultures were prepared as described previously [20] and used after 48 h. Cells were permeabilized and stimulated as previously described [20]. Briefly, the cells were washed twice with Buffer I and then once in Ca^{2+} -free Buffer I. Buffer I comprised (mM): NaCl, 140; KCl, 4; MgCl_2 , 1; CaCl_2 , 1; glucose, 8.3; Hepes, 20 (pH 7.4); Phenol Red, 6 mg/l; and 0.1% (w/v) BSA. The cells were then permeabilized by incubation for 10 min at 37°C in intracellular (IC) buffer containing $3 \mu\text{g}$ of α -toxin/ml and 0.5 mM-EGTA.

Abbreviations used: cAMP, cyclic AMP; $[\text{Ca}^{2+}]_{\text{free}}$, free Ca^{2+} concentration; IBMX, 3-isobutyl-1-methylxanthine; GnRH, gonadotropin-releasing hormone (gonadoliberin); LH, luteinizing hormone (lutropin); PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Me_2SO , dimethyl sulphoxide; pCa, $-\log [\text{Ca}^{2+}]$; EC_{50} , concentration effecting half-maximal response.

* To whom correspondence should be addressed.

Buffer IC comprised (mM): sodium propionate, 140; KCl, 4; Na Pipes, 25 (pH 6.6); MgCl_2 , 6.5; Na_2ATP , 6; Phenol Red, 6 mg/l; 0.1% BSA. After permeabilization, the cell culture plates were cooled on ice for 10 min before equilibration with ice-cold stimulation buffer for 30 minutes. Stimulation buffer comprised buffer IC with Ca-EGTA buffers. Ca-EGTA buffers consisted of EGTA used at 10 mM or 30 mM with different Ca^{2+} concentrations to obtain the indicated $[\text{Ca}^{2+}]_{\text{free}}$, and were prepared as described previously [20]. LH exocytosis was initiated by replacing the stimulation buffer with identical buffer at 37 °C. After 10 minutes the medium was removed and detached cells were pelleted (400 g, 5 min). The supernatant was stored at -20 °C until LH determination. LH was measured by radioimmunoassay as previously described [20], using purified ovine LH and antiserum against ovine LH provided by the NIDDK. Total cellular LH was measured after solubilizing the cells in Nonidet NP40 (1%, v/v). LH released is expressed as a percentage of the total cellular LH present at the beginning of the experiment. IBMX and PMA were added from stock solutions dissolved in Me_2SO , and an equal amount of Me_2SO was added to all the control wells. The highest final concentration of Me_2SO (0.2%) had no effect on LH exocytosis in control experiments.

Stimulation of intact cells and cellular cAMP determination

Anterior-pituitary cells were washed four times (twice briefly and then twice for 10 min) with Buffer I, followed by stimulation in buffer I for 60 min at 37 °C. The medium was collected and processed for LH determination as described above. For cAMP extraction, cells were dissolved in 0.4 ml of 0.1 M-HCl, which was neutralized with 0.1 ml of 100 mM-Tris/NaOH (pH 13) before cAMP determination by radioimmunoassay (Amersham kit no. TKR 342).

Data presentation

All data are representative of experiments performed three to five times: means $\pm$ S.E.M. of triplicate determinations are shown. Error bars were omitted when smaller than the dimensions of the symbol. ANOVA and the modified Student's *t* test were used to evaluate statistical significance.

RESULTS

cAMP-stimulated LH exocytosis

In permeabilized sheep anterior-pituitary cells cAMP stimulated LH exocytosis, with half-maximal LH release at 30 μM -cAMP (Fig. 1). LH exocytosis from intact cells was unaffected by cAMP over a similar concentration range (results not shown). cAMP stimulated LH exocytosis within 5 min and stimulation was sustained for at least 30 min (Fig. 2). This differs from Ca^{2+} -stimulated LH exocytosis in permeabilized cells, which is transient, with no further exocytosis evident after 20 min [20]. The presence of the cyclic nucleotide phosphodiesterase inhibitor IBMX (0.25 mM) decreased the EC_{50} of cAMP from 30 μM to 10 μM (Fig. 1). When used alone, or in combination with IBMX, cGMP (300 μM) did not stimulate LH exocytosis (Table 1). This result suggests that cGMP does not have a role in mediating acute GnRH-stimulated LH exocytosis, a finding which differs from an earlier study in intact cells [21]. It also indicates that IBMX enhances cAMP-stimulated LH exocytosis by inhibiting cAMP hydrolysis rather than cGMP hydrolysis. All subsequent experiments were conducted in the presence of 0.25 mM-IBMX, a concentration which caused optimal enhancement of cAMP-stimulated LH exocytosis without increasing basal LH release (results not shown). Neither cAMP nor IBMX affected permeabilization by α -toxin, as measured by leakage of phosphorylated 2-deoxy[^3H]glucose metabolites (results not shown).

Fig. 1. Effect of cAMP, with or without IBMX, on LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-Ca-EGTA (pCa 7) and cAMP at the indicated concentration alone (○) or in the presence of 0.25 mM-IBMX (●). Exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was determined.

ATP-dependence of cAMP-stimulated LH exocytosis

Both Ca^{2+} - and phorbol-ester-stimulated LH exocytosis are dependent on the presence of millimolar concentrations of MgATP [20]. We therefore investigated the MgATP-dependence of cAMP-stimulated exocytosis. Permeabilized cells equilibrated at 0 °C for 90 min in the absence of MgATP to allow for intracellular depletion of MgATP [20] did not release LH in response to cAMP (Fig. 3). The ability of cAMP to stimulate LH exocytosis was restored by the addition of millimolar MgATP (Fig. 3).

Effect of cAMP on Ca^{2+} -stimulated LH exocytosis

Since a rise in intracellular $[\text{Ca}^{2+}]_{\text{free}}$ occurs in response to GnRH [22–24], we investigated the effect of cAMP on Ca^{2+} -stimulated LH exocytosis. cAMP caused a modest increase in the sensitivity of LH exocytosis to Ca^{2+} , shifting the EC_{50} for Ca^{2+} from pCa 5.6 to pCa 5.9, but had little effect at high $[\text{Ca}^{2+}]_{\text{free}}$ (Fig. 4). Although cAMP-stimulated LH exocytosis was much decreased at very low $[\text{Ca}^{2+}]_{\text{free}}$ (Fig. 4, inset), some stimulation was still evident at pCa 8 and pCa 9 (Table 2).

Fig. 2. Time course of cAMP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-Ca-EGTA (pCa 7) alone (○) or with 100 μM -cAMP (●). Exocytosis was initiated by replacing with identical buffer at 37 °C, which was exchanged at 5 min intervals. The values at each time point represent the LH released during the preceding 5 min period. The zero-time points represent the rate of LH release (per 5 min) during the cold equilibration period.

Table 1. Effects of cGMP on LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min with stimulation buffer containing 10 mM-Ca-EGTA (pCa 7) and the indicated additions. Exocytosis was initiated by replacing with identical buffer at 37 °C, and LH release after 10 min was determined. Results are means ± S.E.M.: * not significantly different from control; ** significantly different from control (*P* < 0.001).

Treatment	LH release (%)
Control	2.50 ± 0.11
cGMP (300 μM)	2.90 ± 0.10*
IBMX (0.25 mM)	2.78 ± 0.32*
cGMP (300 μM)	2.96 ± 0.33*
+ IBMX (0.25 mM)	7.04 ± 0.22**

Fig. 3. ATP-dependence of cAMP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 90 min in stimulation buffer containing 10 mM-Ca-EGTA (pCa 7) and the indicated MgATP concentration alone (○) or with cAMP (100 μM) plus IBMX (0.25 mM) (●). Exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was measured.

Fig. 4. Effect of cAMP on Ca²⁺-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with 30 mM-Ca-EGTA at the indicated [Ca²⁺]_{free} alone (○) or in the presence of cAMP (100 μM) plus IBMX (0.25 mM) (●). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was determined. In the inset the same protocol was used, except that the stimulation-buffer pH was higher (7.1 rather than 6.6) to allow adequate buffering of [Ca²⁺]_{free} down to pCa 9.

Table 2. cAMP-stimulated LH exocytosis at low [Ca²⁺]_{free}

Permeabilized cells were equilibrated at 0 °C for 30 min with stimulation buffer containing 30 mM-Ca-EGTA at pCa 9 or 8 and the indicated additions. Exocytosis was initiated by replacing with identical buffer at 37 °C, and LH release after 10 min was determined. The results (means ± S.E.M.) of *n* independent experiments, each performed in triplicate, were combined: * significantly different from control (*P* < 0.05).

Treatment	LH released (%)
(a) pCa 9 (<i>n</i> = 3)	
Control	3.0 ± 0.4
cAMP (100 μM)	4.2 ± 0.4*
+ IBMX (0.25 mM)	
(b) pCa 8 (<i>n</i> = 5)	
Control	2.3 ± 0.3
cAMP (100 μM)	3.9 ± 0.4*
+ IBMX (0.25 mM)

Fig. 5. Effect of PMA on cAMP-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-Ca-EGTA (pCa 7), IBMX (0.25 mM) and the indicated cAMP concentrations alone (○) or in the presence of 100 nM-PMA (●). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was determined.

Fig. 6. Effect of cAMP on the PMA dose-response curve

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-Ca-EGTA (pCa 7), IBMX (0.25 mM) and the indicated PMA concentrations alone (○) or in the presence of 30 μM-cAMP (●). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was determined.

Fig. 7. Ca^{2+} -dependence of cAMP-plus-PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM-Ca-EGTA with the indicated $[\text{Ca}^{2+}]_{\text{free}}$ with the following additions: ○, none; ●, cAMP (30 μM) plus IBMX (0.25 mM); □, PMA (100 nM); ■, cAMP (30 μM) plus IBMX (0.25 mM) plus PMA (100 nM). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and the LH released after 10 min was determined.

Fig. 8. Effects of GnRH on cAMP production and LH exocytosis in intact cells

Cells at a density of three pituitaries per six-well plate were stimulated for 1 h at 37 °C in Buffer I with IBMX (0.25 mM) and the indicated GnRH concentrations, after which LH release and cellular cAMP were determined as described in the Materials and methods section.

Effect of phorbol ester on cAMP-stimulated LH exocytosis

Because there is evidence that GnRH activates PKC [1], we examined the combined effects of the PKC-activating phorbol ester PMA and cAMP on LH exocytosis. PMA (100 nM) dramatically enhanced cAMP-stimulated LH exocytosis by both decreasing the EC_{50} for cAMP from 10 μM to 3 μM and increasing the maximum LH response 5–7-fold (Figs. 5 and 6). This synergistic interaction was present at low concentrations of PMA (EC_{50} = 10 nM; Fig. 6), and was further enhanced when the free Ca^{2+} concentration was increased over the physiological range (Fig. 7).

GnRH-stimulated cAMP production in intact cells

In the presence of IBMX, GnRH stimulated LH release from intact cells with EC_{50} = 1 nM (Fig. 8). Under the same conditions,

cellular cAMP content was increased by GnRH with EC_{50} = 30 nM (Fig. 8).

DISCUSSION

The aims of this study were, firstly, to establish whether cAMP could directly stimulate acute LH exocytosis independently of its effect on cytosolic Ca^{2+} and, secondly, to examine how cAMP interacts with other second-messenger pathways. Since cAMP can increase intracellular $[\text{Ca}^{2+}]_{\text{free}}$ [17,18], the ability of cAMP analogues or forskolin to stimulate LH exocytosis in intact cells may be a consequence of increased $[\text{Ca}^{2+}]_{\text{free}}$. In the present experiments we used permeabilized cells in which the intracellular $[\text{Ca}^{2+}]_{\text{free}}$ is strongly buffered with high concentrations of EGTA. The results establish conclusively that cAMP can stimulate LH exocytosis directly without any change in the $[\text{Ca}^{2+}]_{\text{free}}$. The rapid effect of cAMP (evident at 5 min) indicates that cAMP could play a role in acute GnRH-stimulated LH exocytosis during which stores of previously synthesized LH are released. Previous studies using intact rat pituitary cells have demonstrated stimulatory effects of cAMP analogues or forskolin on LH secretion, but stimulation was generally observed only after 1–4 h [4,7,19,25]. The effect of cAMP analogues may be delayed because of their slow entry into the cell or because, in the rat, the effects of cAMP are mediated by an increase in LH synthesis [25]. We have found that, in intact sheep anterior-pituitary cells, cAMP analogues, while not detectably stimulating LH exocytosis alone, do synergistically enhance phorbol-ester-stimulated LH exocytosis during a 15–20 min stimulation (P. Kaye & J. S. Davidson, unpublished work).

As is the case with Ca^{2+} - and phorbol-ester-stimulated LH exocytosis [20], cAMP-stimulated LH exocytosis required millimolar MgATP concentrations. Since cAMP-dependent protein kinase (protein kinase A) is saturated at micromolar ATP concentrations [26], this suggests that at least one ATP-dependent step distinct from protein kinase A is involved in cAMP-stimulated exocytosis. This step appears to be common to Ca^{2+} -, PKC- and cAMP-stimulated exocytosis.

The ability of raised $[\text{Ca}^{2+}]_{\text{free}}$ to stimulate an increase in intracellular cAMP concentrations by activating Ca^{2+} /calmodulin-dependent adenylate cyclases [27,28] raises the question as to whether Ca^{2+} stimulates LH exocytosis indirectly through effects on cAMP. This is clearly not the case since high $[\text{Ca}^{2+}]_{\text{free}}$ stimulated much more extensive LH exocytosis than did maximally effective cAMP concentrations, and Ca^{2+} and cAMP, when used together, showed a synergistic interaction in stimulating LH exocytosis.

High concentrations of cAMP were necessary to stimulate exocytosis (EC_{50} 30 μM in the absence of IBMX), and the maximal effect was small compared with stimulation with Ca^{2+} or phorbol ester. These findings might cast doubt on the physiological relevance of cAMP as a mediator of exocytosis. However, in the presence of the PKC-activating phorbol ester PMA, cAMP stimulated extensive LH exocytosis at low micromolar concentrations, well within the range expected *in vivo*, and this effect was enhanced by increasing the $[\text{Ca}^{2+}]_{\text{free}}$ over the physiological range. Since GnRH stimulation results in an increase in intracellular $[\text{Ca}^{2+}]_{\text{free}}$ [22–24] and probably activates PKC [1], even a small increase in the intracellular cAMP concentration would result in significant enhancement of LH exocytosis. Because PMA has previously been shown to be capable of stimulating adenylate cyclase activity [27,29], it could be argued that the effects of phorbol ester result from an increase in cAMP production. However, our finding that very low concentrations of cAMP dramatically enhance PMA-stimulated LH exocytosis suggest that cAMP does not mediate the effect of PMA.

The stimulation of cAMP production by GnRH in intact anterior-pituitary cells supports a role for cAMP in GnRH-stimulated LH exocytosis. The different dose-dependence of GnRH-stimulated LH exocytosis and GnRH-stimulated cAMP production (EC_{50} 1 nM and 30 nM respectively) is not unexpected, since theoretical modelling of second-messenger cascades predicts a shift in agonist dose-dependence to lower agonist concentrations when responses further down the cascade are examined [30].

In conclusion, we have shown that cAMP is able directly to stimulate LH exocytosis independently of Ca^{2+} and that cAMP synergistically enhances PMA- and Ca^{2+} -stimulated LH exocytosis. These findings suggest that cAMP plays a major role in GnRH-stimulated LH exocytosis, through its synergistic interactions with PKC and Ca^{2+} .

We gratefully acknowledge support by grants from the South African Medical Research Council, the Stella and Paul Loewenstein Trust, the National Cancer Association, and the Nellie Atkinson and Becker bequests of the University of Cape Town.

REFERENCES

- Huckle, W. R. & Conn, P. M. (1988) *Endocr. Rev.* **9**, 387–394
- Borgeat, P., Chavancy, G., Dupont, A., Labrie, F., Arimuru, A. & Schally, A. V. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2677–2681
- Kaneko, T., Saito, S., Oka, H., Oda, T. & Yanaihara, N. (1973) *Metab. Clin. Exp.* **22**, 77–80
- Makino, T. (1973) *Am. J. Obstet. Gynaecol.* **115**, 606–614
- Bonney, R. C. & Cunningham, F. J. (1977) *Mol. Cell. Endocrinol.* **7**, 233–244
- Kercret, H., Benoist, L. & Duval, J. (1977) *FEBS Lett.* **83**, 222–224
- Naor, Z., Koch, Y., Chobsiang, P. & Zor, U. (1975) *FEBS Lett.* **58**, 318–321
- Conn, P. M., Morrel, D. V., Dufau, M. L. & Catt, K. J. (1979) *Endocrinology (Baltimore)* **104**, 448–453
- Sen, K. K. & Menon, K. M. J. (1979) *Biochem. Biophys. Res. Commun.* **87**, 221–228
- Benoist, L., Le Dafniet, M., Rotsztein, W. H., Besson, J. & Duval, J. (1981) *Acta Endocrinol. (Copenhagen)* **97**, 329–337
- Smith, M. A., Perrin, M. H. & Vale, W. W. (1982) *Endocrinology (Baltimore)* **111**, 1951–1957
- Capponi, A. M., Aubert, M. L. & Clayton, R. N. (1984) *Life Sci.* **34**, 2139–2144
- Hoshi, T., Garber, S. S. & Aldrich, R. W. (1988) *Science* **240**, 1652–1655
- Osterrieder, W., Brum, W., Hescheler, J., Trautwein, W., Flockerzi, V. & Hofmann, F. (1982) *Nature (London)* **298**, 576–578
- Curtis, B. M. & Catterall, W. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2528–2532
- Armstrong, D. & Eckert, R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2518–2522
- Luini, A., Lewis, D., Guild, S., Corda, D. & Axelrod, J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8034–8038
- Lau, K. & Bourdau, J. E. (1989) *J. Biol. Chem.* **264**, 4028–4032
- Cronin, M. J., Evans, W. S., Hewlett, E. L. & Thorner, M. O. (1984) *Am. J. Physiol.* **246**, E44–E51
- van der Merwe, P. A., Millar, R. P., Wakefield, I. K. & Davidson, J. S. (1989) *Biochem. J.* **264**, 901–908
- Snyder, G., Naor, Z., Fawcett, C. P. & McCann, S. M. (1978) *Endocrinology (Baltimore)* **107**, 1627–1633
- Clapper, D. L. & Conn, P. M. (1985) *Biol. Reprod.* **32**, 269–278
- Chang, J. P., McCoy, E. E., Graeter, J., Tasaka, K. & Catt, K. J. (1986) *J. Biol. Chem.* **261**, 9105–9108
- Limor, R., Ayalon, D., Capponi, A. M., Childs, G. V. & Naor, Z. (1987) *Endocrinology (Baltimore)* **120**, 497–503
- Bourne, G. A. & Baldwin, D. M. (1987) *Am. J. Physiol.* **253**, E290–E295
- Walsh, D. A. & Krebs, E. G. (1973) *Enzymes 3rd Ed.* **8**, 555–581
- Brostrom, M. A., Brotman, L. A. & Brostrom, C. O. (1982) *Biochim. Biophys. Acta* **721**, 227–235
- Minocherhomjee, A. M., Shattuck, R. L. & Storm, D. R. (1988) in *Calmodulin* (Cohen, P. & Klee, C. B., eds.), pp. 249–264, Elsevier, Amsterdam
- Summers, S. T. & Cronin, M. J. (1986) *Biochem. Biophys. Res. Commun.* **135**, 276–281
- Strickland, S. & Loeb, J. N. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1366–1370

Received 26 February 1990/12 June 1990; accepted 26 June 1990