0026-895X/87/040488-09$02.00/0

MOLECULAR PHARMACOLOGY, 32:488-496

Activators of Protein Kinase C and Cyclic AMP-Dependent Protein Kinase Regulate Intracellular Calcium Levels Through Distinct Mechanisms in Mouse Anterior Pituitary Tumor Cells

TERRY REISINE and SIMON GUILD

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (T.R.) and Department of Pharmacology, University of Glasgow, Glasgow, United Kingdom (S.G.)

Received March 30, 1987; Accepted July 7, 1987

SUMMARY

The effects of the protein kinase C activator, phorbol myristate acetate (PMA), on cytosolic calcium levels and adrenocorticotropin (ACTH) release from the mouse anterior pituitary tumor cell line, AtT-20, were compared to those induced by the hormone, corticotropin-releasing factor (CRF), a stimulant of cAMP-dependent protein kinase activity. Cytosolic calcium levels were measured using the fluorescence probe Quin 2. PMA induced a time- and concentration-dependent rise in cytosolic calcium levels and ACTH release from AtT-20 cells that was blocked by verapamil and nifedipine, antagonists of voltage-regulated calcium channels, and tetraethylammonium (TEA), a K^+ channel antagonist. The inactive phorbol ester, 4-phorbol 12,13-didecanoate, did not alter cytosolic calcium levels or ACTH release. Several minutes after the initial stimulation of calcium influx by PMA, cytosolic calcium levels returned to basal levels despite the continued presence of the phorbol ester. A short pretreatment (2–4 min) of AtT-20 cells with PMA abolished the ability of K^+, CRF, and forskolin to raise intracellular calcium levels. These findings indicate that phorbol esters induce a secondary inhibition of calcium influx after an initial stimulation. In contrast to the effects of PMA, CRF induced a sustained rise in cytosolic calcium levels and did not reduce the subsequent stimulation of calcium influx by K^+ or PMA. CRF-stimulated calcium influx was blocked by verapamil but not TEA. The ability of CRF to elevate cytosolic calcium levels was mediated by cAMP-dependent protein kinase because the insertion of a synthetic peptide inhibitor of cAMP-dependent protein kinase activity into AtT-20 cells attenuated the ability of CRF and forskolin but not PMA to raise cytosolic calcium levels. The results suggest that activators of protein kinase C and cAMP-dependent protein kinase regulate intracellular calcium levels in AtT-20 cells through different mechanisms.

Protein kinase C is a calcium- and lipid-dependent enzyme (1) that is widely distributed throughout the nervous system as well as peripheral organs (2). This enzyme has been proposed to have an important role in the process of stimulus-secretion coupling (3–5). The mechanisms by which protein kinase C regulates hormone and neurotransmitter release are not clearly established. In adrenal chromaffin cells and *Apylsia* neurons, protein kinase C activation increases calcium conductance and calcium influx (6, 7). Stimulation of calcium influx is known to trigger hormone or neurotransmitter secretion. However, Rane and Dunlap (8) have observed that phorbol esters and diacylglycerol, activators of protein kinase C, reduce calcium conductance in chicken embryonic dorsal root ganglion neurons. Furthermore, Harris *et al.* (9) have shown that phorbol esters diminish depolarization-induced influx of $^{45}Ca^{2+}$ in PC12 cells. Thus, phorbol esters may exert opposing actions on calcium influx depending on the cell type.

In the present study, we have examined the effect of phorbol esters on the levels of cytosolic calcium and hormone release from a tumor cell line of the mouse anterior pituitary, AtT-20. We show that phorbol esters stimulate a rapid increase of intracellular free calcium in AtT-20 cells that is dependent on calcium influx. This effect appears to lead to the stimulation of ACTH secretion. The stimulation is followed by an inhibition of hormone- and depolarization-induced calcium influx and may result from a direct blockade of calcium conductance channels. The contrasting actions of phorbol esters on the same cell type appear to be mediated by protein kinase C and may involve the regulation of different ionic conductance channels.

Besides protein kinase C, cAMP-dependent protein kinase also regulates hormone and neurotransmitter release (10).

This work was supported by a grant-in-aid for the American Heart Association and by National Institutes of Health Grants DK37407 and GM34782.

ABBREVIATIONS: ACTH, adrenocorticotropin; CRF, corticotropin-releasing factor; PMA, phorbol myristate acetate; PDD, 4-phorbol 12,13-didecanoate; TEA, tetraethylammonium; PKI, cAMP-dependent protein kinase inhibitor peptide; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salts solution; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; N-CAM, neuronal cell adhesion molecule; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Quin 2, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-amino-quinoline; Quin 2AM, the acetomethoxy ester of Quin 2.

cAMP is believed to evoke ACTH secretion from AtT-20 cells by enhancing calcium conductance and influx so as to raise cytosolic calcium levels (11–13). In the present study, it is shown that cAMP-dependent protein kinase activation leads to an increase in cytosolic calcium levels in AtT-20 cells through a mechanism distinct from that induced by phorbol esters. Furthermore, unlike protein kinase C, cAMP does not produce a secondary inhibition of calcium influx. The two protein kinases, therefore, stimulate different cascades of molecular events to regulate cellular calcium levels and ACTH secretion.

Experimental Procedures

Materials

Forskolin, Quin 2AM, and ionomycin were purchased from Calbiochem-Behring (San Diego, CA). CRF was obtained from Peninsula Laboratories (San Carlos, CA). PMA, PDD, TEA, and KCl were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM (4500 mg of glucose/liter) and HBSS were obtained from Grand Island Biological Co. (Grand Island, NY).

Cell Culture Techniques

Mouse AtT-20/D16-16 tumor cells were grown and subcultured in DMEM with 10% fetal calf serum as previously described (14). Cells were plated in 75-cm^2 culture flasks at an initial density of 1,000,000 cells/flask and were used 4–5 days after subculturing (60–80% confluency).

Quin 2 Fluorescence Studies

The cells were washed twice by resuspension/centrifugation (500 × *g*, 5 min) in 10 ml of DMEM supplemented with 0.25% (w/v) (DMEM/BSA). The cells were resuspended in 10 ml of DMEM/BSA and incubated with 50 μM (final concentration) of the acetomethoxy methylester of Quin 2 (Quin 2 AM) for 15 min at 37° in a shaking water bath. The cells were subsequently washed twice by resuspension/centrifugation in DMEM/BSA and, at the end of the second centrifugation, the cells were resuspended in 6 ml of DMEM/BSA. The cell suspension was divided into 1-ml aliquots and placed in an atmosphere of 5% CO_2 at 37° for 5 min. The tubes were then sealed and stored at room temperature until they were used in the fluorescence determinations.

A 1-ml aliquot of the loaded cells was added to 10 ml of HBSS containing 0.02% (w/v) BSA (HBSS/BSA), at 37°. This sample was centrifuged, the supernatant was discarded, and the cells were resuspended in 2 ml of HBSS/DMEM. This suspension was then transferred to a quartz fluorimeter cuvette, and the cell fluorescence was determined in a temperature-controlled (at 37°) fluorescence spectrophotometer. The excitation wavelength was 339 nm (slit width 5 nm) and the emission wavelength was 492 nm (slit width 10 nm). The cells were continually stirred by a magnetic stirrer. At the beginning of each experiment, the formation of Quin 2-free acid was verified by identifying the characteristic emission peak at 492 nm. If this was not evident, the batch of cells was discarded.

The cell suspensions were then challenged by test agents (PMA, PDD, K^+, ionomycin, CRF, forskolin, gramicidin, TEA, verapamil and nifedipine). The fluorescence signal was continuously measured for at least 10 min following the challenge with any of the agents. At the end of each experiment, 20 μg of ionomycin were added to the cells (to equilibrate intracellular calcium levels with the calcium in the extracellular medium), and the resulting fluorescence signal, which corresponds to a calcium concentration of 1 mM and was designated maximal fluorescence (F_{max}), was recorded. The cells were then sonicated with a Branson Sonicator (10 W, 5 sec). Enough EGTA was added to reduce the concentration of free calcium below 1 nM. The resulting fluorescence signal was designated minimal fluorescence (F_{min}). These values of F_{max} and F_{min} were used to determine the concentration of free calcium corresponding to the experimental fluorescence signals by the equation detailed by Tsien *et al.* (15). The change in autofluorescence after sonication was estimated in unloaded cells and the calibration values were adjusted accordingly.

Pretreatments

PMA. Plated AtT-20 cells were exposed to DMEM containing either PMA (100 nM), PDD (100 nM), or an equivalent amount of the solvent dimethyl sulfoxide (control, 0.001%) for 2 hr. After this time, the cells were washed three times and prepared for the fluorescence studies.

PKI. The procedures employed for the insertion of the inhibitor of cAMP-dependent protein kinase into AtT-20 cells using targeted liposomes have been described extensively elsewhere (16, 17). Polyclonal anti-N-CAM antibodies were obtained from a rabbit immunized with an antigen preparation purified as previously described (18) on a monoclonal H-28 anti-N-CAM immunoaffinity column from adult mouse brain. The liposome synthesis protocol has been described (16, 17). However, in the present study, the synthetic 20-amino acid-containing peptide PKI, described by Cheng *et al.* (19), was encapsulated into the liposomes instead of the partially purified preparation of rabbit muscle inhibitor protein. In the liposome preparation, 10 mg of the pure PKI was dissolved in water and mixed with the lipids. In previous studies (17), it was shown that 6% of the commercially available protein kinase inhibitor was encapsulated into the liposomes. Control liposomes were prepared by the same procedure with the exception that PKI was omitted. The liposome preparation was coupled to *Staphylococcus aureus* protein A as described by Leserman *et al.* (20).

For the PKI pretreatment studies, the AtT-20 cells were washed twice with 3 ml of DMEM containing 25 mM Hepes and 2% fetal calf serum. The cells were then incubated with 2 ml of medium with anti-N-CAM antibody (final dilution 1:300) for 1 hr at 25° as previously described (16, 17). The cells were then washed twice and incubated for 3 hr, at 37° in 10% CO_2, with 3 ml of medium with liposomes coupled to protein A. Cells were exposed to liposomes either containing or lacking PKI. At the end of this pretreatment time, the medium was removed and fresh DMEM was applied to the cells. The cells were detached from the culture flask by repeated pipetting of the medium over the plated cells. Two separate groups of cells resulted from these procedures: 1) PKI containing liposome-treated cells, and 2) cells treated with liposomes lacking PKI. Each of these cell populations was prepared separately for the measurement of their cytosolic calcium concentration.

Preparation on PKI

The PKI was synthesized for us by Dr. Daniele Moiuier, Centre d'Immunologie, CNRS-INSERM, Marseille, France. The peptide (Thr-Thr-Tyr-Ala-Asp-Phe-Ileu-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ileu-His-Asp) described by Cheng *et al.* (19) was synthesized by manual solid phase synthesis according to the method of Baramy and Merrifield (21). The resin (0.4 mg/g) was obtained from Peninsula; the protected amino acids were from Bachem (Bubendorf, Switzerland) or Beckman (Palo Alto, CA). The side chains of Asp, Ser, and Thr were protected by benzyl groups and the phenol of Tyr by oligochlorobenzyl. Deprotection and cleavage from the resin were done by treatment with liquid hydrogen fluoride at 0° for 1 hr. Purification was performed by gel filtration on a Bio-Gel P-2 (150 × 2.5 cm) column in 0.2 M acetic acid, followed by high pressure liquid chromatography using a C_{18} μBondapak column and a trifluoroacetic acid acetonitrile gradient as described by Cheng *et al.* (19). Purity was assessed by high voltage paper electrophoresis, amino acid composition after hydrolysis in 6 N HCl for 20 hr, and sequence analysis. The peptide was 95% pure and had the expected amino acid composition and sequence.

To test the biological activity of the synthetic PKI, the ability of the peptide to inhibit cAMP-dependent protein kinase activity was determined. The kinase reaction was performed according to the procedure

of Glass *et al.* (22). Briefly, the reaction mixture consisted of 62.5 mM (2-*N*-morpholino)ethanesulfonic acid (pH 6.9), 0.4 mM [^{32}P]ATP (New England Nuclear), 2 mM magnesium acetate, 0.3 mg/ml BSA, 3 mM 2-mercaptoethanol, 0.1 mM cAMP, 1 μg of the catalytic subunit of cAMP-dependent protein kinase (Sigma), 100 μg of Histone Type IIS (Sigma), and 2 μg of the synthetic PKI in a total volume of 80 μl. The reaction was initiated by the addition of the kinase and was run for 10 min at 30°. The reaction was terminated by placing the mixture onto Whatman P81 paper squares (2 × 2 cm) and immediately washing with water. The squares were then dried and the radioactivity was determined in a scintillation counter.

ACTH Release

ACTH release was measured as previously described (14). The radioimmunoassay sensitivity was 3 pg of ACTH immunoreactivity.

cAMP Assay

The cAMP studies were performed as previously described (14). cAMP immunoreactivity was detected using a radioimmunoassay.

Statistics

The traces of the fluorescence signals in the figures depict the results from representative experiments performed upon a single preparation of cells. The traces indicate accuracy of experimental technique and the magnitude of the biological phenomena under observation, but not biological variability. The biological variability of an observed phenomenon was determined by reproducing an experimental protocol at least five times. The data obtained from these multiple determinations were averaged, and the mean ± standard error is presented in the legends of the figures. The calcium concentrations, determined from the observed fluorescence changes, depicted in each figure are the average calcium concentration in the cell suspension. Student's *t* test (paired, two-tailed) was used for all statistical comparisons. Differences with $p < 0.05$ were considered significant.

Results

In the present study, we have used the fluorescence dye Quin 2 to measure cytosolic calcium levels in AtT-20 cells. Depolarization of AtT-20 cells with 60 mM potassium induced a rapid and pronounced increase in cytosolic calcium levels (Fig. 1). In contrast, the peptide hormone CRF, which activates adenylate cyclase and cAMP-dependent protein kinase in AtT-20 cells (16, 23, 24), produced a delayed rise in cytosolic calcium that plateaued at about 50% above basal levels (Fig. 1). The CRF-induced increase was maintained for up to 10 min (not shown). Similar patterns of increase in cytosolic calcium, as seen with CRF, are obtained with forskolin, a direct activator of adenylate cyclase, and 8-bromo-cAMP (12, 13, 24). The elevation of cytosolic calcium levels in response to CRF and K^+ was due to an increase in calcium influx into AtT-20 cells since it was blocked by the calcium channel antagonists, verapamil (Fig. 2) and nifedipine (not shown). These agents also prevented the ACTH release response to CRF and K^+ (13).

PMA increases cytosolic calcium levels. The phorbol ester, PMA (1 μM), which stimulates protein kinase C activity in AtT-20 cells (25), induced a more rapid elevation of cytosolic calcium levels than did CRF (Fig. 1). The stimulation reached maximal levels within 1 min. The rise in cytosolic calcium was dependent on the concentration of the phorbol ester used, with significant increases occurring with 0.1 nM PMA or higher (Fig. 3). (EC_{50} value = 0.3 ± 0.05 nM, n = 5). PMA had a similar potency for evoking ACTH release from AtT-20 cells (Fig. 4). (EC_{50} value = 0.6 ± 0.1 nM, n = 5). Increases in cytosolic

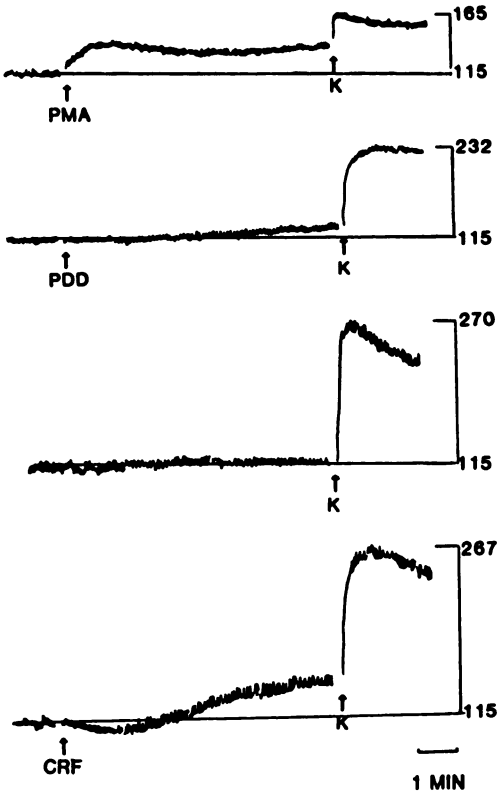

Fig. 1. PMA increases cytosolic calcium levels and blocks the effects of potassium in stimulating calcium influx. Intracellular calcium levels were determined using Quin 2 as described under Experimental Procedures. Representative traces of the fluorescence changes in batches of cells of individual experiments are presented. The value of the *far right* refer to the calculated calcium concentrations (nM) under basal and stimulated (maximal increases) conditions. These experiments were repeated at least six different times with similar results. *K* refers to K^+ (60 mM). PMA and PDD were added to a final concentration of 1 μM and CRF was added to a 100 nM final concentration.

calcium levels and ACTH release were also observed with the relatively water-soluble phorbol ester, phorbol 12,13-dibutyrate (not shown). The inactive phorbol ester, PDD, which does not affect protein kinase C activity in AtT-20 cells, did not alter cytosolic calcium levels (Fig. 1) or ACTH release (Fig. 4). The elevation in cytosolic calcium in response to PMA was blocked by verapamil (Fig. 2). Similar results were obtained with nifedipine (not shown). Both calcium channel antagonists prevented PMA from evoking ACTH release (Fig. 4).

PMA could enhance calcium influx directly by activating the calcium channel, as shown by the actions of cAMP-dependent protein kinase in heart cells (26). Alternatively, PMA could act indirectly by depolarizing the cell membrane. Depolarization of the plasma membrane might be expected to lead to an increase in either frequency or duration of opening of the voltage-regulated calcium channels. To depolarize the cell membrane, phorbol esters may block potassium conductance channels as suggested from studies on photoreceptor cells and hippocampal neurons (27, 28). Some potassium conductance channels can be blocked by TEA. Patch clamp studies by Wong and Adler (29) have shown that TEA inhibits calcium-activated potassium conductance channels in AtT-20 cells. Addition of TEA to AtT-20 cells induced a rapid, small [basal calcium = 112 ± 5 nM, TEA (10 mM) = 153 ± 8 nM, n = 10] and transient increase in cellular calcium (Fig. 5). Lower concentrations of

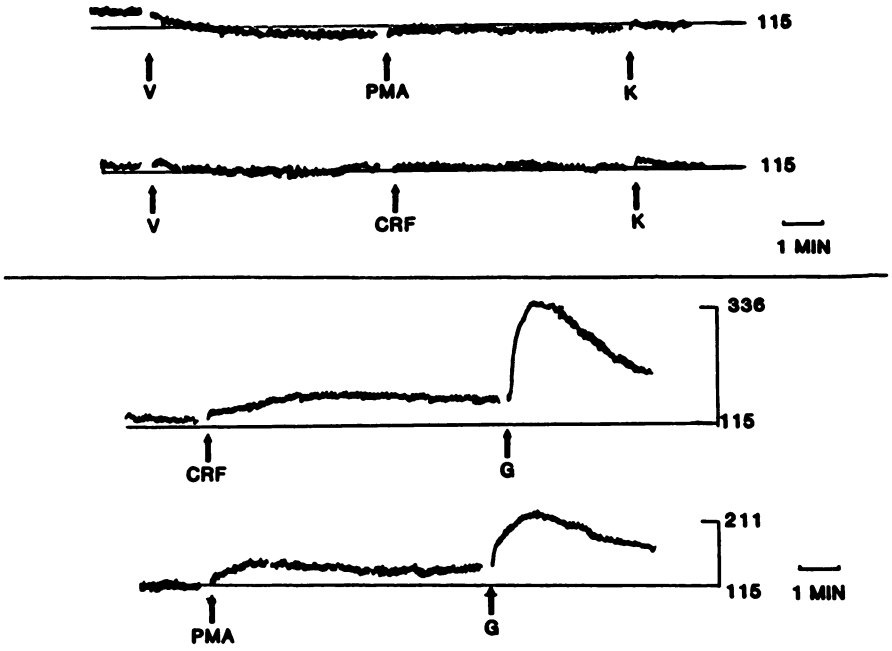

Fig. 2. Modulation of cytosolic calcium levels by verapamil and gramicidin. *Top*, AtT-20 cells were continuously exposed to verapamil (*V*) (1 μM) and stimulated with either PMA (1 μM), CRF (100 nM), or K^+ (60 mM). Representative traces of five separate experiments are presented. *Bottom*. Cells were exposed either to CRF (100 nM) or PMA (1 μM) for 5 min and then treated with gramicidin (*G*) (1 μM). On the *far right* are the basal (*lower value*) and maximal cytosolic calcium concentrations (nM) observed in these representative traces of individual experiments. Experiments were repeated at least five different times. The means ± SE of the maximal calcium concentration (nM) were: basal = 115 ± 5, CRF = 161 ± 7, CRF ± G = 342 ± 15, G = 332 ± 8, PMA = 159 ± 7, PMA ± G = 205 ± 14.

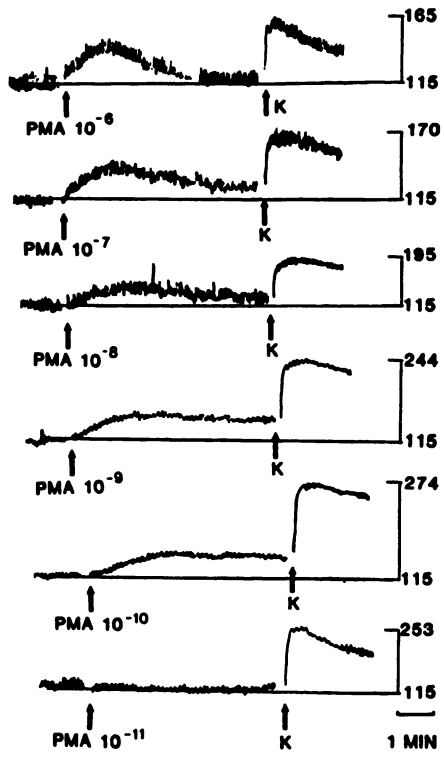

Fig. 3. Dose dependency of PMA to modulate cytosolic calcium levels. Different concentrations (M) of PMA were applied to individual batches of AtT-20 cells. Five min after the addition of PMA, K^+ (*K*, 60 mM) was applied. Continuous traces of the fluorescence changes of representative experiments are presented. The calculated intracellular calcium levels (nM) under basal (*lower value* of *trace*) and stimulated (*upper value* in *trace*) conditions are represented on the *far right*. Experiments were repeated at least five different times. The means ± SE of the maximal calcium concentrations (nM) are: basal = 115 ± 5; PMA, 1 μM = 150 ± 5, 100 nM = 148 ± 7, 10 nM = 153 ± 6, 1 nM = 143 ± 7, 100 pM = 127 ± 6, 10 pM = 117 ± 6.

TEA (0.1–1 mM) produced little effect on cytosolic calcium levels (not shown). Following TEA application to the AtT-20 cells, the addition of PMA to AtT-20 cells was no longer able to raise cytosolic calcium levels (Fig. 5). In contrast, CRF, potassium (Fig. 5), and ionomycin (not shown) induced the same maximal rise in intracellular free calcium in control cells as in cells preexposed to 10 mM TEA.

Blockade of calcium influx. PMA, in addition to increasing intracellular calcium levels by itself, prevented potassium from raising cytosolic calcium levels. High (1 μM) PMA concentrations blocked the subsequent rise in cytosolic calcium induced by 60 mM potassium to approximately 30% of control (Fig. 1). The ability of lower potassium concentrations to elevate intracellular calcium concentrations was also attenuated by PMA treatment (not shown). This inhibition was dependent on the concentration of PMA, with half-maximal blockade at a 5 nM concentration of the phorbol ester (Fig. 3). PDD did not significantly reduce the cellular response to potassium (Fig. 1). The blockade of the response of AtT-20 cells to K^+ by PMA was rapid, with a significant inhibition occurring after only 1 min of PMA exposure to the cells (Fig. 6). Addition of PMA to cells after exposure to K^+ reduced cytosolic calcium to basal levels (Fig. 7). In contrast, K^+ alone maintained intracellular calcium levels above basal concentrations.

PMA also blocked the rise in cytosolic calcium induced by CRF (basal = 115 ± 5 nM, CRF = 169 ± 8 nM, PMA followed 5 min later by CRF = 129 ± 8 nM $n\pm$ = 5). This inhibition was not due to an uncoupling of CRF receptors from adenylate cyclase because CRF stimulated cAMP formation to the same extent in both control and phorbol ester-treated cells (not shown). Furthermore, PMA prevented the rise in cytosolic calcium in response to forskolin, a direct activator of adenylate cyclase (basal calcium, 120 ± 10 nM; forskolin, 50 μM, 165 ± 15 nM; PMA followed 5 min later by forskolin, 125 ± 20 nM, n = 6). PMA, however, did not reduce the ability of the calcium ionophore, ionomycin, to elevate intracellular free calcium levels (not shown).

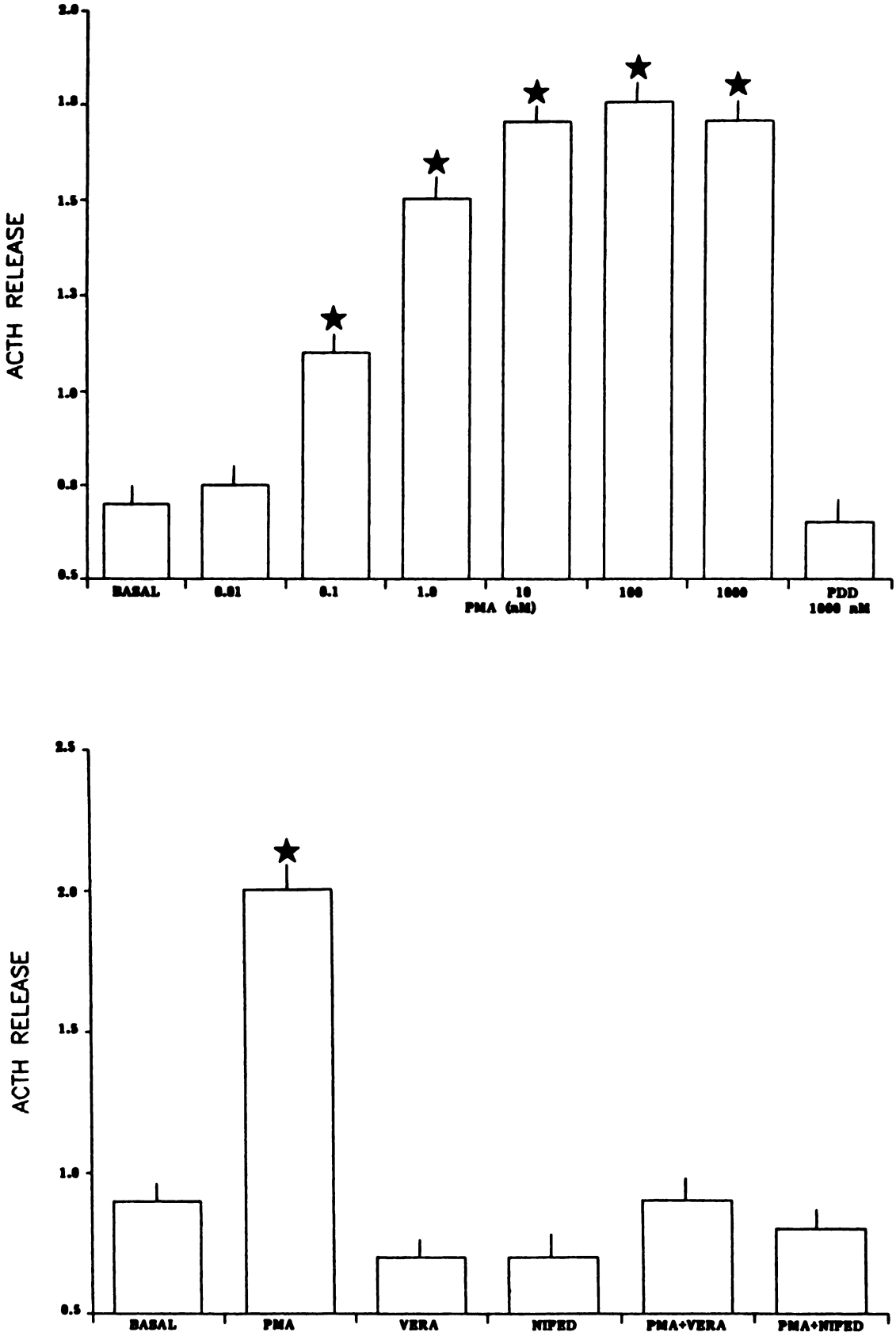

Fig. 4. Effect of PMA on ACTH release. *Top.* AtT-20 cells were plated in 24-well Costar plates at an initial density of 100,000 cells/well and grown in DMEM with 10% fetal calf serum. Four to 5 days after seeding, the growth medium was removed and the cells were exposed to DMEM with 25 mM Hepes (pH 7.4) for 1 hr. The medium was removed and the cells were treated with varying concentrations of PMA or 1 μM PDD. One hr after the drug addition, an aliquot of medium was removed and analyzed for ACTH immunoreactivity. The values represent the mean ± standard error of five different experiments done in triplicate wells. Values are presented as ng of ACTH immunoreactivity per well. ★, $p < 0.05$, significantly different from basal ACTH release. *Bottom*, Cells were exposed to either PMA (1 μM), verapamil (*VERA*, 1 μM), nifedipine (*NIFED*, 100 nM), or their combination for 1 hr. ACTH immunoreactivity was measured as described under Experimental Procedures. Values represent the mean ± standard error of five separate experiments done in triplicate wells and are expressed as ng of ACTH immunoreactivity per well. ★, $p < 0.05$, significantly different from basal release using a Student's *t* test.

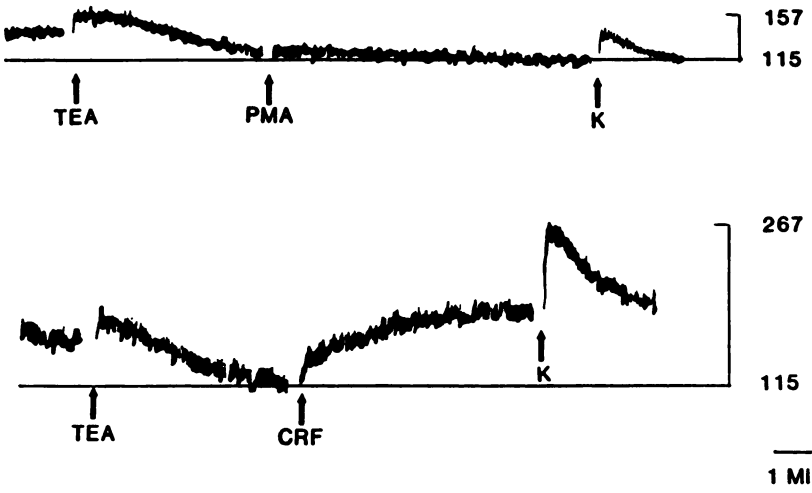

Fig. 5. Effect of TEA on cytosolic calcium levels in AtT-20 cells. AtT-20 cells were exposed to TEA (10 mM) and then stimulated with either PMA (1 μM) or CRF (100 nM). Three to 5 min afterwards, K^+ (60 mM) was applied to the cells. Presented are representative traces of experiments that were repeated six different times. The mean ± SE maximal calcium concentrations (nM) observed following TEA application were: basal = 118 ± 7, PMA = 115 ± 6, PMA ± K = 135 ± 9, CRF = 171 ± 8, CRF ± K = 275 ± 8.

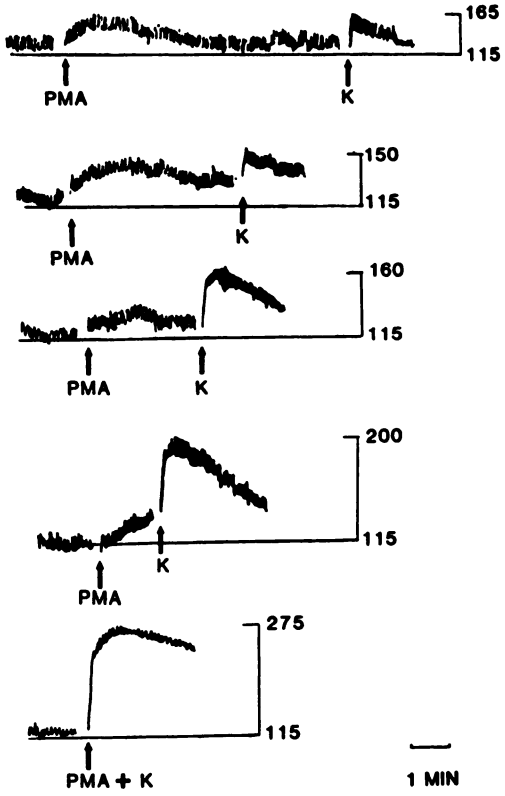

Fig. 6. Time course for PMA inhibition of K^+-stimulated rises in cytosolic calcium. AtT-20 cells were exposed to PMA (1 μM) for varying times and then stimulated with K^+ (60 mM). Representative traces of individual experiments are presented. The cytosolic calcium concentrations in response to K^+ are depicted on the *far right*. The *bottom trace* depicts the effect of the simultaneous addition of PMA and K^+. The experiments were repeated five times. The mean ± SE maximal calcium concentrations (nM) followng K^+ stimulation after various times (min) of PMA treatment are: basal = 115 ± 5; PMA pretreatment time 0 min = 268 ± 11, 1 min = 205 ± 8, 2 min = 165 ± 9, 3 min = 155 ± 7, 4 min = 159 ± 8.

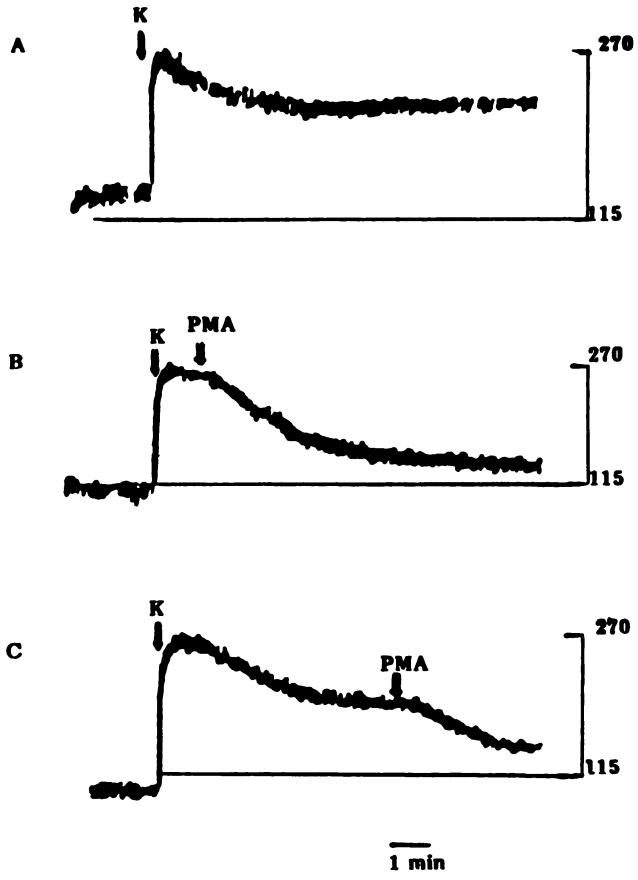

Fig. 7. The effect of the addition of PMA to K^+-stimulated cells. AtT-20 cells were stimulated with 60 mM K^+. After an immediate increase in cytosolic calcium levels, calcium concentrations gradually declined and plateaued to a level above basal. A. A representative trace of an individual experiment depicting the effect of K^+ on cytosolic calcium levels. The action of PMA (1 μM), applied to the cells 1 min (B) and 4 min (C) (when cytosolic calcium levels plateaued following K^+ challenge; see A) after K^+ challenge, on cytosolic calcium levels is also presented. These experiments were repeated at least five different times with results similar to those depicted in the figure.

To test whether the inhibitory effects of PMA were dependent on changes in membrane potential, gramicidin was applied to AtT-20 cells (Fig. 2). Gramicidin is an ionophore that depolarizes cells by equilibrating sodium ion and potassium ion gradients across the plasma membrane. Gramicidin induced a very rapid and pronounced rise in cytosolic calcium levels in AtT-20 cells (Fig. 2). The maximal increase was attenuated 50% by PMA pretreatment (Fig. 2). A similar inhibitory effect occurred when lower gramicidin concentrations were employed (not shown). CRF did not modify the effect of gramicidin in raising cytosolic calcium levels (Fig. 2).

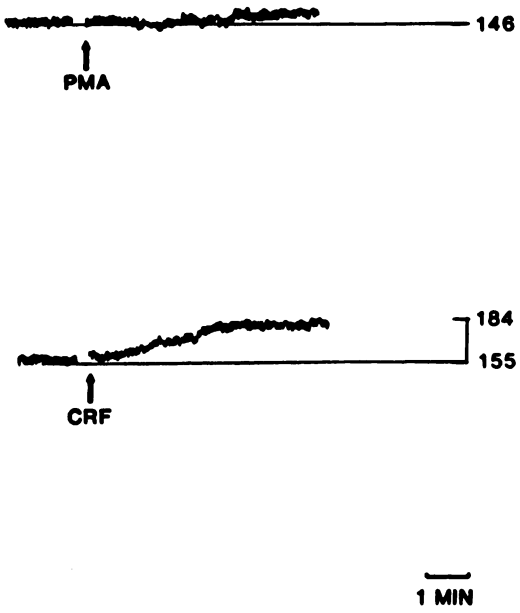

Fig. 8. PMA desensitization. AtT-20 cells were exposed to PMA (100 nM) for 2 hr. The cells were then washed and prepared for the Quin 2 studies. The representative traces obtained from pretreated cells either challenged with PMA (1 μM) or CRF (100 nM) are presented. These experiments were performed on six different groups of cells. The mean ± SE maximal calcium levels (nM) are: basal = 148 ± 7, PMA = 151 ± 6, CRF = 188 ± 9.

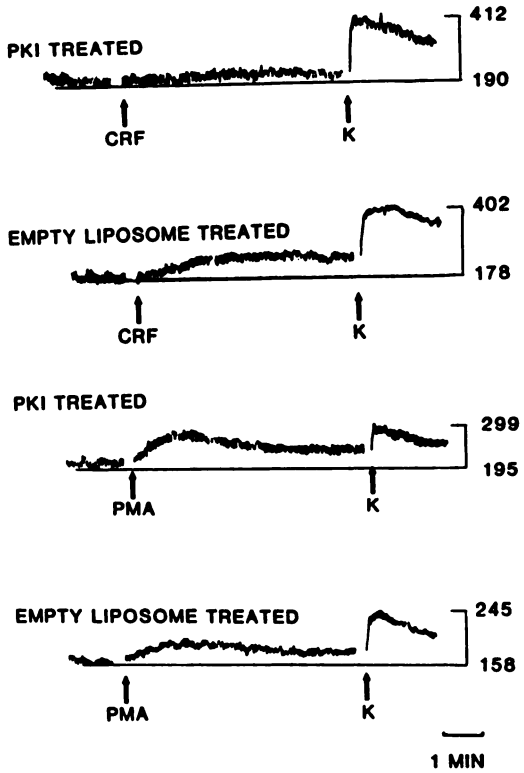

Fig. 9. Effect of PKI on cytosolic calcium levels. AtT-20 cells were treated with liposomes either containing (*PKI TREATED*) or lacking (*EMPTY LIPOSOME TREATED*) the PKI. These cells were then stimulated with either CRF (100 nM), PMA (1 μM), or K^+ (60 mM). These are representative traces of individual experiments. Experiments were repeated on 10 different batches of cells. The mean ± SE maximal calcium concentrations (nM) are: Empty Liposome Treated, basal = 165 ± 12, CRF = 221 ± 11, PMA = 219 ± 13, CRF ± K = 400 ± 30; PKI Treated, basal = 170 ± 10, CRF = 180 ± 11, PMA = 228 ± 9, CRF ± K = 413 ± 25.

PMA desensitization. Previous studies (30) have shown that numerous receptor-coupled cellular responses to phorbol esters are rapidly and specifically desensitized during continuous phorbol ester treatment. In AtT-20 cells, phorbol ester desensitization is believed to be a two-step process (30). Initially, the ability of phorbol ester to evoke ACTH release declines, and then the cytosolic phorbol ester-binding sites down-regulate. Pretreatment of AtT-20 cells with PMA (100 nM) for 2 hr totally abolished the ability of PMA (1 μM) to raise cytosolic calcium levels (Fig. 8). However, CRF (100 nM) was still able to increase intracellular calcium concentrations in the phorbol ester-desensitized cells (Fig. 8).

Selective blockade of cAMP-mediated effects. In addition to protein kinase C, cAMP-dependent protein kinase is also involved in stimulating calcium influx into AtT-20 cells. CRF and forskolin, which activate cAMP-dependent protein kinase in AtT-20 cells (23), induce a maximal increase in cytosolic calcium levels of similar magnitude to that of active phorbol esters, and, like PMA, their effects are blocked by verapamil (13). To demonstrate that CRF and forskolin increase calcium influx through an activation of cAMP-dependent protein kinase, we (16, 17) developed a procedure for inserting the Walsh inhibitor of cAMP-dependent protein kinase into intact AtT-20 cells using a liposome technique. The insertion of this protein preparation into the cells resulted in the blockade of CRF-, forskolin, and 8-bromo-cAMP-induced ACTH release and forskolin-induced protein phosphorylation (16, 17, 31). In contrast, potassium- and phorbol ester-stimulated ACTH release and phorbol ester-induced protein phosphorylation were not modified by the protein kinase inhibitor treatment. Thus, it was possible to selectively block cAMP-dependent protein kinase activity in these cells without affecting the cellular responses to phorbol ester.

Recently, Cheng *et al.* (19) characterized a 20-amino acid peptide (PKI) which was very effective and selective in blocking cAMP-dependent protein kinase activity. This peptide was synthesized for us (>95% pure as assessed by high voltage paper electrophoresis), and we found it to block cAMP-dependent protein kinase activity. Encapsulation of this peptide into liposomes and its insertion into AtT-20 cells reduced the rise in cytosolic calcium in response to CRF to 30% of control values (Fig. 9). The maximal rise in cytosolic calcium induced by forskolin was similarly attenuated in cells treated with the PKI (control basal calcium = 170 ± 13 nM; forskolin, 50 μM = 219 ± 11 nM; PKI-treated basal = 175 ± 12 nM; forskolin, 50 μM = 189 ± 11 nM, n = 6). In contrast, PMA as well as potassium induced the same magnitude increase in cytosolic calcium in cells treated with empty and PKI-filled liposomes (Fig. 9). The concentration-dependent increase in cytosolic calcium levels elicited by potassium also was not modified by the PKI treatment (not shown). Furthermore, the calcium ionophore, ionomycin, elevated intracellular free calcium concentrations to the same extent in empty and PKI-filled liposome-treated cells (not shown).

Discussion

Modulation of ionic conductance may be a major mechanism by which protein kinase C regulates hormone and neurotransmitter release from electrically excitable cells. By enhancing

calcium conductance and calcium influx, protein kinase C activation could raise intracellular free calcium above the threshold needed to evoke hormone or neurotransmitter secretion. Depolarization of the cell membrane leading to enhanced calcium influx may be an essential step in the cascade of events that protein kinase C initiates to trigger the release of bioactive substances.

Phorbol esters have been shown to depolarize hippocampal neurons and photoreceptor cells (16, 27, 28). The mechanism involved in this depolarization is believed to be a blockade of potassium channels. Blockade of some K^+ channels with TEA increased cytosolic calcium levels and prevented PMA from further stimulating calcium influx into AtT-20 cells. Since TEA probably depolarizes AtT-20 cells, its addition to these cells may have removed the means by which phorbol esters could depolarize the plasma membrane to open voltage-gated calcium channels and elevate intracellular calcium concentrations. This is further suggested from the studies in which prior depolarization of AtT-20 cells with K^+ prevented PMA from further increasing cytosolic calcium levels. The blockade of PMA-induced calcium influx by TEA was selective, as TEA did not modify the ability of K^+, CRF, or ionomycin to raise cytosolic calcium levels. Thus, phorbol esters may inhibit a class of TEA-sensitive K^+ channels in AtT-20 cells to depolarize the cell and stimulate calcium influx.

The rise in cytosolic calcium in response to PMA was directly related to the ability of the phorbol ester to evoke ACTH secretion. The potency of PMA to induce ACTH release and raise cytosolic calcium levels was similar to the affinity of cytosolic receptors (proposed to be protein kinase C) in AtT-20 cells to bind radiolabeled PMA and for the phorbol ester to stimulate protein kinase C activity in these cells (25, 30). Furthermore, an inactive phorbol ester did not modify calcium levels or ACTH release from AtT-20 cells. The ability of PMA to evoke ACTH release and raise cytosolic calcium levels was blocked by calcium channel antagonists, suggesting that both responses involve an increase in calcium influx. The increases in cytosolic calcium induced by PMA preceded the stimulation of ACTH release induced by the phorbol ester (32), which is consistent with the concept that elevated calcium influx triggers hormone secretion.

Whereas phorbol esters initially stimulated calcium influx into AtT-20 cells, several minutes after the PMA application, cytosolic calcium returned to basal levels. This refractoriness could be due to desensitization of phorbol ester-binding sites and protein kinase C. However, several minutes after PMA addition, AtT-20 cells were no longer able to respond to K^+, CRF, or forskolin by increasing cytosolic calcium levels. Pretreatment of AtT-20 cells for 2 hr with PMA, which down-regulates phorbol ester-binding sites and abolishes PMA-stimulated calcium influx and ACTH release (30), did not modify CRF-stimulated calcium influx or ACTH secretion (30). Thus, the rapid (within minutes) inhibition of CRF- and K^+-stimulated calcium influx following PMA treatment does not appear to be explained by phorbol ester desensitization. In fact, phorbol esters most likely reduce K^+- and CRF-stimulated calcium influx through an inhibition of calcium conductance. Rane and Dunlap (8) have shown that phorbol esters inhibit calcium conductance of dorsal root ganglion neurons. The ability of PMA to suppress gramicidin-induced calcium influx would suggest that protein kinase C activation causes a secondary blockade of calcium conductance in AtT-20 cells.

The question arises as to how phorbol esters induce their biphasic stimulation followed by an inhibition of calcium influx in AtT-20 cells. Phorbol esters may rapidly inhibit K^+ conductance in AtT-20 cells to depolarize the cell membrane. This depolarization might be expected to enhance the opening of voltage-regulated calcium channels to increase calcium influx. Subsequently, calcium conductance may be reduced by phorbol esters. Protein kinase C activation may catalyze a series of sequential phosphorylation events to block first, K^+ and, then, calcium channels. Alternatively, all the relevant protein phosphorylation may occur simultaneously, yet those phosphorylation events may induce their biological response at different rates. To our knowledge, the mechanism by which protein kinase C can coordinately regulate different ionic conductance channels in the same cell has not been reported in any cell type. In fact, to our knowledge, phorbol esters have not been previously reported to affect multiple ionic conductance channels or to both stimulate and inhibit calcium influx in the same cell type.

In addition to protein kinase C, cAMP-dependent protein kinase also regulates cytosolic calcium levels in AtT-20 cells and the secretion of ACTH (11–13, 16, 17). This enzyme is activated in both AtT-20 cells and normal corticotrophs by the hormone, CRF, which is the most potent and effective natural stimulant of ACTH release (11). To examine the role of cAMP-dependent protein kinase in mediating CRF-evoked ACTH secretion, we (16, 17) inserted the Walsh inhibitor of cAMP-dependent protein kinase into AtT-20 cells, using a liposome technique. In cells loaded with this inhibitor protein, the ACTH release response to CRF, forskolin, and 8-bromo-cAMP was blocked (16, 17). Furthermore, the ability of CRF to stimulate calcium influx is also blocked by insertion of a synthetic peptide inhibitor of cAMP-dependent protein kinase into these cells. These findings are consistent with the hypothesis that CRF stimulates cAMP-dependent protein kinase to enhance calcium influx to trigger ACTH release.

The mechanisms by which cAMP and protein kinase C regulate calcium levels in AtT-20 cells appear to be distinct. The rise in cytosolic calcium in response to CRF was not blocked by TEA. This may suggest that cAMP-dependent protein kinase does not act through the same population of K^+ channels as protein kinase C to modify membrane potential. Unlike phorbol esters, cAMP may in fact act to directly stimulate calcium channels. In support of this notion, Luini *et al.* (13) have shown that 8-bromo-cAMP increases the frequency of opening of calcium channels in voltage-clamped patches of intact AtT-20 cells.

Once CRF increases cytosolic calcium levels in AtT-20 cells, this effect is maintained for as long as the cells are exposed to the stimulant. This pattern contrasts to the biphasic effect exhibited by PMA. Furthermore, neither CRF nor forskolin inhibit K^+-induced increases in calcium influx into AtT-20 cells. These findings indicate that cAMP-dependent protein kinase does not cause a secondary inhibition of calcium influx.

Although CRF and PMA induce different patterns of effect on cytosolic calcium in AtT-20 cells, both agents have been shown to evoke a sustained increase in ACTH release (32). Because CRF- and PMA-evoked ACTH release is calcium dependent, it is conceivable that the initial burst of calcium

influx stimulated by both agents is sufficient to cause long-term increases in hormone secretion. Such findings may indicate that corticotrophs need not be continuously stimulated by hormones to sustain a prolonged elevation of ACTH release. Recent studies (33), in fact, have shown that the synthesis of ACTH in AtT-20 cells is elevated by CRF and PMA long after the cells have become desensitized to the acute effects of these secretagogues. The molecular mechanisms of such long-term effects on the expression and release of ACTH are unknown.

Our findings suggest that the activation of protein kinase C and cAMP-dependent protein kinase stimulates calcium influx through different cascades of events in AtT-20 cells. Determination of the nature of these different events will require identification of the cellular phosphoproteins regulated by these two kinases and the ionic conductance channels modulated by these phosphorylation events.

Acknowledgments

We would like to thank Drs. J. Munk and T. Abrams for their helpful discussions on this manuscript and Dr. D. Moiuier for synthesizing the PKI for us.

References

1. Takai, Y., A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori, and Y. Nishizuka. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254**:3692–3695 (1979).
2. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.)* **308**:693–695 (1984).
3. Pozzan, T., G. Gatti, N. Dozio, L. Vicentini, and J. Meldolesi. Calcium-dependent and -independent release of neurotransmitters from PC12 cells: a role for protein kinase C activation. *J. Cell. Biol.* **99**:628–638 (1984).
4. Publicover, S. Stimulation of spontaneous transmitter release by the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate, an activator of protein kinase C. *Brain Res.* **333**:185–187 (1985).
5. Shapira, R., S. Silberberg, S. Ginsburg, and R. Rahaminoff. Activation of protein kinase C augments evoked transmitter release. *Nature (Lond.)* **325**:58–60 (1987).
6. DeRiemer, S., J. Strong, K. Albert, P. Greengard, and L. Kaczmarek. Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature (Lond.)* **313**:313–316 (1985).
7. Wakade, A., R. Malhotra, and T. Wakade. Phorbol esters facilitate (45)Ca accumulation and catecholamine secretion by nicotine and excess K^+ but not by muscarine in rat adrenal medulla. *Nature (Lond.)* **321**:698–700 (1986).
8. Rane, S., and K. Dunlap. Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA* **83**:184–188 (1986).
9. Harris, K., S. Kongsamut, and R. Miller. Protein kinase C mediated regulation of calcium channels in PC12 pheochromocytoma cells. *Biochem. Biophys. Res. Commun.* **134**:1298–1305 (1986).
10. Nestler, E., and P. Greengard. *Protein Phosphorylation in the Nervous System.* John Wiley & Sons, Inc., New York (1984).
11. Axelrod, J., and T. Reisine. Interaction among the stress hormones. *Science (Wash. D.C.)* **224**:452–459 (1984).
12. Guild, S., Y. Itoh, J. Kebabian, A. Luini, and T. Reisine. Forskolin enhances basal and potassium evoked release from normal and malignant pituitary tissue: the role of calcium. *Endocrinology* **118**:268–279 (1986).
13. Luini, A., D. Lewis, S. Guild, D. Corda, and J. Axelrod. Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells. *Proc. Natl. Acad. Sci. USA* **82**:8034–8038 (1985).
14. Reisine, T. Somatostatin desensitization: loss of the ability of somatostatin to inhibit cyclic AMP accumulation and ACTH release. *J. Pharmacol. Exp. Ther.* **229**:14–20 (1984).
15. Tsien, R., T. Pozzan, and T. Rink. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. *J. Cell. Biol.* **94**:325–334 (1982).
16. Reisine, T., J. Barbet, G. Rougon, and H.-U. Affolter. Incorporation of the inhibitor of cyclic AMP-dependent protein kinase into anterior pituitary tumor cells using liposomes blocks CRF-induced adrenocorticotropin hormone release and synthesis. *Proc. Natl. Acad. Sci. USA* **82**:8261–8265 (1985).
17. Reisine, T., G. Rougon, and J. Barbet. Liposome delivery of cyclic AMP dependent protein kinase inhibitor into intact cells: specific blockade of cyclic AMP-mediated adrenocorticotropin release from mouse anterior pituitary tumor cells. *J. Cell. Biol.* **102**:1630–1637 (1986).
18. Rougon, G., H. Deagostini-Bazin, M. Hirn, and C. Goridis. Tissue and developmental stage specific forms of a neural cell surface antigen linked to differences in glycosylation of a common polypeptide. *EMBO J.* **1**:1239–1244 (1982).
19. Cheng, H., S. Van Patten, A. Smith, and D. Walsh. An active twenty amino-acid residue peptide derived from the inhibitor protein of the cAMP dependent protein kinase. *Biochem. J.* **231**:655–661 (1985).
20. Leserman, L., J. Barbet, F. Kourilsky, and J. Weinstein. Targeting of cells by fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature (Lond.)* **288**:602–604 (1980).
21. Baramy, G., and R. Merrifield. Solid-phase peptide synthesis, in *Peptide Analysis, Synthesis, and Biology.* (E. Gross and J. Meienhofer, eds.) Vol 2. Academic Press, New York, 1–284 (1980).
22. Glass, D., R. Masaracchia, J. Feramisco, and B. Kemp. Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem.* **87**:566–575 (1978).
23. Litvin, Y., R. Pasmaniter, N. Fleischer, and J. Erlichman. Hormonal activation of the cAMP dependent protein kinases in AtT-20 cells. Preferential activation of the protein kinase I by corticotropin releasing factor, isoproterenol and forskolin. *J. Biol. Chem.* **259**:10296–10303 (1984).
24. Reisine, T., and S. Guild. Pertussis toxin blocks somatostatin inhibition of calcium mobilization and desensitizes somatostatin receptors. *J. Pharmacol. Exp. Ther.* **235**:551–557 (1985).
25. Zatz, M., L. Mahan, and T. Reisine. Translocation of protein kinase C in anterior pituitary tumor cells. *J. Neurochem.* **48**:106–110 (1987).
26. Reuter, H. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (Lond.)* **301**:569–575 (1983).
27. Madison, D., R. Malenka, and R. Nicoll. Phorbol esters block a voltage-sensitive chloride current in hippocampal pyramidal cells. *Nature (Lond.)* **321**:695–697 (1986).
28. Farley, J., and S. Auerbach. Protein kinase C activation induces conductance changes in *Hermissenda* photoreceptors like those seen in associative learning. *Nature (Lond.)* **319**:220–223 (1986).
29. Wong, B., and M. Adler. Asymmetrical sensitivity of TEA blockade of calcium-activated potassium channels in clonal anterior pituitary cells. *Soc. Neurosci. Abstr.* **11**:790 (1985).
30. Phillips, M., and S. Jaken. Specific desensitization of tumor-promoting phorbol esters in mouse pituitary cells. *J. Biol. Chem.* **258**:2875–2881 (1983).
31. Rougon, G., J. Barbet, H.-U. Affolter, and T. Reisine. cAMP-dependent protein kinase activation and protein phosphorylation in AtT-20 cells. *Soc. Neurosci. Abstr.* **11**:1093 (1985).
32. Heisler, S. 12-*O*-Tetradecanoyl-phorbol-13-acetate-induced ACTH secretion in pituitary tumor cells. *Eur. J. Pharmacol.* **98**:177–183 (1984).
33. Affolter, H.-U., and T. Reisine. Corticotropin releasing factor increases proopiomelancortin messenger RNA in mouse pituitary tumor cells. *J. Biol. Chem.* **260**:15477–15481 (1985).

Send reprint requests to: Dr. Terry Reisine, Department of Pharmacology, University of Pennsylvania School of Medicine, 36th Street and Hamilton Walk, Philadelphia, PA 19104.