Role of Protein Kinase C in Catecholamine Secretion from Digitonin-permeabilized Bovine Adrenal Medullary Cells*

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The effects of staurosporine and K-252a, potent inhibitors of protein kinases, and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on catecholamine secretion and protein phosphorylation in digitonin-permeabilized bovine adrenal medullary cells were investigated. Staurosporine and K-252a (0.01-10 μM) did not cause large changes in catecholamine secretion evoked by Ca2+ in digitonin-permeabilized cells whereas these compounds strongly prevented TPA-induced enhancement of catecholamine secretion in a concentration-dependent manner. Incubation of digitonin-permeabilized cells with [γ-32P]ATP resulted in 32Pi incorporation into a large number of proteins, detected as several major bands and darkened background in autoradiograms. Ca2+ and TPA increased phosphorylation of these proteins. Staurosporine and K-252a markedly inhibited Ca2+-induced and TPA-induced increases in protein phosphorylation as well as basal (0 Ca2+) protein phosphorylation in digitonin-permeabilized cells. Long term treatment (24 h) of adrenal medullary cells with 1 μM TPA markedly decreased total cellular protein kinase C activity to about 5.3% of control. Pre-treatment of the cells with 1 μM TPA strongly inhibited the TPA-induced enhancement of catecholamine secretion whereas it did not cause large changes in total cellular catecholamine amounts, Ca2+-induced catecholamine secretion, and cAMP-induced enhancement of catecholamine secretion from digitonin-permeabilized cells. From these results we conclude that protein kinase C plays a modulatory role in catecholamine secretion rather than being essential for initiating catecholamine secretion.

EXPERIMENTAL PROCEDURES

Culture of Adrenal Medullary Cells—Bovine adrenal medullary cells were isolated by sequential digestion of adrenal medullary slices with collagenase as reported previously (14), purified by differential plating (15), and maintained as monolayer cultures in Eagle's minimal essential medium containing 10% heat-inactivated calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.3 μg/ml amphotericin B, and 10 μg/ml cytosine arabinoside. The cells were plated at a density of 5 x 10⁵ cells/well in 24-well cluster plates and 1.5 x 10⁶ cells/well in 96-well cluster plates. For the experiments of down-regulation of protein kinase C 3-day-cultured cells were incubated for 24 h in the presence of TPA or vehicle (Me2SO).

Catecholamine Secretion from Permeabilized Cells—The culture cells were washed with 0.5 ml of permeabilizing medium consisting of 150 mM monosodium glutamate and 10 mM PIPES (pH 7.0) and then permeabilized for 6 min with 0.5 ml of permeabilizing medium containing 10 μM digitonin and 1 mM EGTA. The permeabilized cells were then stimulated for the indicated time in 0.5 ml of permeabilizing medium containing 2 mM MgATP, 5 mM EGTA, various amounts of CaCl2, and vehicle (Me2SO) or test compounds. Free Ca2+ concentrations in the medium were measured with a Ca2+-sensitive electrode constructed using the neutral ligand ETHE 1001 (16). In some cases, described in the figure legends, the cells were preincubated for 15 min of exocytotic catecholamine secretion (4-11). Lee and Holz examined extensively the effects of various phorbol ester analogs on protein phosphorylation and catecholamine secretion, and they reported that certain phorbol esters, which could substitute for diacylglycerol and similarly increased the affinity of protein kinase C for Ca2+ in vitro, also enhanced protein phosphorylation and Ca2+-induced catecholamine secretion in adrenal medullary cells (4). However, it is still controversial whether protein kinase C is essential for initiating exocytotic catecholamine secretion (8-11). Tryptsin inhibits a phorbol ester-induced increase in catecholamine secretion without any effect on primary secretion (10). On the other hand, various protein kinase C inhibitors depress both primary secretion and a phorbol ester-induced increase in secretion (8).

Staurosporine and K-252a have been recently reported to be novel and potent inhibitors of protein kinases (12, 13). These compounds nonspecifically inhibit protein kinase C, myosin light chain kinase, cAMP-dependent protein kinase, and cGMP-dependent protein kinase at nanomolar concentrations in vitro. In the present study we examined the effects of staurosporine, K-252a, and TPA on catecholamine secretion and protein phosphorylation in digitonin-permeabilized bovine adrenal medullary cells to elucidate the role of protein kinase C in catecholamine secretion.
with 0.5 ml of permeabilizing medium containing Me2SO or test compounds and then stimulated for 10 min with 0.5 ml of permeabilizing medium containing 10 μM digitonin, 2 mM MgATP, various concentrations of free Ca2+, and Me2SO or test compounds. This medium was then removed and centrifuged at 700 x g for 5 min and the supernatant was assayed for catecholamines. Catecholamines were determined by the ethylenediamine condensation method (17). The experiments were performed at room temperature, between 23 and 28 °C.

**Protein Phosphorylation in Permeabilized Cells**—The culture cells were incubated for 6 min with 0.15 ml of permeabilizing medium containing 10 μM digitonin and 1 mM EGTA and then stimulated for the indicated time in 0.1 ml of permeabilizing medium containing 2 mM [γ-32p]ATP (15-30 Ci/mM, 2 mM MgCl2, various concentrations of free Ca2+, and Me2SO or test compounds. In some cases, described in the figure legends, the cells were preincubated for 15 min with 0.15 ml of permeabilizing medium containing Me2SO or test compounds and then stimulated for 10 min with 0.1 ml of permeabilizing medium containing 10 μM digitonin, 2 mM [γ-32p]ATP, 2 mM MgCl2, various concentrations of free Ca2+, and Me2SO or test compounds. Incubations were stopped by replacing the medium with 70 μl of sodium dodecyl sulfate (SDS) buffer consisting of 2.3% SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), and 1% (v/v) bromophenol blue. Samples were incubated at 100 °C for 5 min and then analyzed for phosphoprotein by SDS-polyacrylamide slab gel electrophoresis in the buffer system of Laemmli (18). Gels were stained with Coomassie Brilliant Blue R, dried, and exposed to Kodak X-OMAT AR-2 film. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograms at 430 nm using a Shimazu chromatogram scanner model CS-930. The molecular marker proteins used were α-lactalbumin (14,100), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase b (94,000).

**Protein Kinase C Assay**—The cells (16-mm wells) were solubilized in 0.4 ml of lysis buffer consisting of 20 mM PIPES (pH 7.0), 0.1% Triton X-100, 10 mM dithiothreitol, and 50 μg/ml leupeptin. Protein kinase C activity in the samples was assayed in a reaction mixture (100 μl) consisting of cell extracts (1:50 diluted), 24 mM PIPES (pH 7.0), 10 mM MgCl2, 50 μM [γ-32p]ATP (0.2-0.5 Ci/tube), 200 μg/ml histone H1 (type III), 50 μg/ml phosphatidyserine, 100 nM TPA, 5 mM EGTA, or 5 mM EGTA-calcium, 2 mM dithiothreitol, 0.02% Triton X-100, and 10 μg/ml leupeptin. After a 10-min incubation at 37 °C the reaction was stopped by the addition of 10 μl of 10 mM MgATP and 100 mM EDTA. 50-μl aliquots were then dropped onto 2.2 x 1.2-cm phosphocellulose paper (Whatman P81), which was washed five times with water. The radioactivity on each piece of paper was determined by scintillation counting. The reaction was linear with respect to time and enzyme concentration under the conditions used.

Because protein kinase C activity was maximal with about 10 nM TPA and did not largely change at around 100 nM TPA used in assay, protein kinase C activity in the samples is not dependent on TPA treatment of the cells.

**Materials**—Stauroporine and K-252a were purchased from Kyowa Medex Co. ETH 1001 was from Fluka Chemie AG. Other chemicals and materials were obtained from commercial sources.

**RESULTS**

Effects of Stauroporine and K-252a on Catecholamine Secretion—TPA activates protein kinase C, which is the only known cellular receptor for this compound (19, 20) and therefore has been used widely as a means of testing whether protein kinase C is involved in cellular events. TPA (1-1,000 nM) increased catecholamine secretion evoked by Ca2+ in digitonin-permeabilized bovine adrenal medullary cells in a concentration-dependent manner but had little effect on basal (0 Ca2+) catecholamine secretion. Enhancement of Ca2+-dependent catecholamine secretion was almost maximal with 100 nM TPA (data not shown). On the other hand, 4α-phorbol 12,13-didecanoate, inactive phorbol ester on protein kinase C, had little effect on catecholamine secretion. These results are in good agreement with the reports that protein kinase C is intimately involved in catecholamine secretion from digitonin-permeabilized cells (4, 6, 7, 9-11). Fig. 1 shows the effects of various concentrations of stauroporine and K-252a on Ca2+- and TPA-dependent catecholamine secretion from digitonin-permeabilized adrenal medullary cells. Stauroporine (0.01-10 μM) caused a little inhibition on catecholamine secretion evoked by 0.95 μM Ca2+ whereas this compound at the same range of concentrations strongly prevented TPA-induced enhancement of catecholamine secretion. Inhibition of TPA-induced enhancement of catecholamine secretion was detectable with as little as 48 nM stauroporine and was half-maximal with about 0.48 μM. Almost complete inhibition was observed with 2.2 μM stauroporine. K-252a had little effect on Ca2+-evoked catecholamine secretion but inhibited TPA-induced enhancement of catecholamine secretion in a concentration-dependent manner (IC50, about 1 μM). Moreover, stauroporine and K-252a did not cause large changes in catecholamine secretion induced by 2.1 μM Ca2+, comparable to that by 0.95 μM Ca2+ plus 100 nM TPA (Fig. 1). Therefore, the possibility that stauroporine and K-252a solely inhibit more vigorous exocytosis is unlikely.

Fig. 2A shows the concentration-dependent enhancement of catecholamine secretion when digitonin-permeabilized cells were incubated with TPA, stauroporine, and K-252a. Ca2+-evoked catecholamine secretion and TPA-induced enhancement of catecholamine secretion continued for about 30 min. K-252a (10 μM) had little effect on time course of catecholamine secretion evoked by Ca2+ but strongly inhibited TPA-induced enhancement of catecholamine secretion. Stauroporine (3 μM) caused a significant inhibition of the time course of catecholamine secretion evoked by Ca2+ but still showed selective inhibition on TPA-induced enhancement of catecholamine secretion during the time course of TPA-induced catecholamine secretion.

Fig. 2B shows the effect of K-252a on catecholamine secretion induced by various concentrations of free Ca2+ in the presence and absence of TPA. TPA (100 nM) increased catecholamine secretion at all Ca2+ concentrations to elicit secretion but had little effect on basal catecholamine secretion. K-252a (10 μM) had little effect on the Ca2+ dose-response relation for catecholamine secretion but strongly inhibited the TPA-induced enhancement of Ca2+-dependent catecholamine secretion.

Fig. 2C shows the effect of the preincubation with TPA and

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**FIG. 1.** Effects of various concentrations of stauroporine and K-252a on Ca2+-evoked catecholamine secretion from digitonin-permeabilized bovine adrenal medullary cells in the presence and absence of TPA. Bovine adrenal medullary cells were preincubated for 6 min in permeabilizing medium containing 10 μM digitonin and 1 mM EGTA and were then stimulated for 10 min in permeabilizing medium containing 2 mM MgATP, with or without Ca2+, various concentrations of protein kinase inhibitors (A, stauroporine; B, K-252a), and 0 or 100 nM TPA. The catecholamine content released into the medium was measured and was expressed as a percentage of the total catecholamine in the cells. Values shown are means ± S.E. of three experiments. X, 0 Ca2+; ●, 0.95 μM Ca2+; ○, 0.95 μM Ca2+ + 100 nM TPA; ■, 2.1 μM Ca2+.
Ca\textsuperscript{2+}- and Phorbol Ester-dependent Protein Phosphorylation

Effects of Staurosporine and K-252a on Protein Phosphorylation—We examined the effects of staurosporine and K-252a on protein phosphorylation in digitonin-permeabilized bovine adrenal medullary cells to investigate whether the inhibitory effects of staurosporine and K-252a on TPA-induced enhancement of catecholamine secretion are caused by a suppression of protein phosphorylation. Incubation of digitonin-permeabilized cells with various concentrations of free Ca\textsuperscript{2+} resulted in 32P\textsuperscript{+} incorporation into a large number of proteins, detected as several major bands of 100, 89, 59, 39, 26, 18, and 16 kDa and darkened background in autoradiograms (Fig. 3). This result is consistent with the report that active phorbol esters inhibit the release of protein kinase C molecules from the digitonin-permeabilized cells (21). On the other hand, staurosporine (3 \mu M) had no inhibitory effect on the Ca\textsuperscript{2+} dose-response relation for catecholamine secretion. It is unclear whether the inhibitory effect of staurosporine on Ca\textsuperscript{2+}-induced catecholamine secretion reflects a small role for protein kinase C in Ca\textsuperscript{2+}-induced catecholamine secretion in the absence of phorbol ester or whether the compound has a nonspecific deleterious effect on the cells. Staurosporine at the same concentration almost completely inhibited the TPA-induced enhancement of catecholamine secretion in response to various concentrations of Ca\textsuperscript{2+}. Preincubation with 10 \mu M K-252a following the protocol in Fig. 2C resulted in a small increase in Ca\textsuperscript{2+}-evoked catecholamine secretion and a slight leftward shift of the Ca\textsuperscript{2+} dose-response curve (data not shown). This slight increase in catecholamine secretion by the preincubation with K-252a was not explored. K-252a in a marked increase in phosphorylation of many proteins although the degree of phosphorylation of these proteins although the degree of inhibition depends on each protein. These results are in good agreement with the experiments that staurosporine and K-252a nonselectively inhibit various protein kinases in vitro (12, 13).

TPA (100 nM) caused a small but significant increase in protein phosphorylation (Fig. 3). Preincubation of intact cells with 100 nM TPA for 15 min before permeabilization resulted in a marked increase in phosphorylation of many proteins (Fig. 4). Staurosporine (3 \mu M) and K-252a (10 \mu M) inhibited not only basal and Ca\textsuperscript{2+}-induced protein phosphorylation but also TPA-induced protein phosphorylation. The relative intensity of the bands was quantitated by densitometric tracing of the autoradiograms at 430 nm using a Shimazu chromatogram scanner model CS-930. The TPA-induced increase in the intensity of the band at 59 kDa was 66 ± 12%, 23 ± 7%, and 5 ± 4% (means ± S.E. of three experiments) at 0.1, 1.0, and 10 \mu M staurosporine, respectively, and 78 ± 11%, 42 ± 15%, and 8 ± 5% at 0.1, 1.0, and 10 \mu M K-252a, respectively. These dose-response curves are similar to the dose-response curves of these compounds on the TPA-induced increase in catecholamine secretion but not to those on Ca\textsuperscript{2+}-induced catecholamine secretion in the absence of TPA (Fig. 1). The results suggest that selective inhibition of staurosporine and
containing 2 mM \[\gamma-^{32}P\]ATP (15-30 pCi/well), 2 mM MgCl\(_2\), indicated concentrations of free Ca\(^{2+}\), 0 or 100 nM TPA, and in the absence or presence of protein kinase inhibitors (A, 3 \(\mu\)M staurosporine; B, 10 \(\mu\)M K-252a). Incubations were stopped by replacing the medium with SDS buffer. Samples were subjected to SDS-polyacrylamide (7.5-15% gradient) gel electrophoresis and autoradiography as described under "Experimental Procedures."

K-252a on TPA-induced enhancement of catecholamine secretion is caused by a suppression of protein phosphorylation.

**Effect of Long Term Treatment of TPA**—In many cell types prolonged protein kinase C stimulation with active phorbol esters leads to down-regulation of protein kinase C with a loss of enzyme activity and the protein itself as detected by immunoprecipitation and binding of \[^{3}H\]phorbol 12,13-dibutyrate (9, 22, 23). However, it is still controversial whether down-regulation of protein kinase C results in a decrease in primary secretion of catecholamine in bovine adrenal medullary cells (9, 11). We also studied the effect of the long term treatment of TPA on catecholamine secretion and protein kinase C activity in bovine adrenal medullary cells (Fig. 5). 24-h pretreatment of the cells with 1 \(\mu\)M TPA markedly decreased total cellular protein kinase C activity from a control level of 6.45 \(\pm\) 0.22 to 0.34 \(\pm\) 0.06 (nmol of ATP/10 min/5 \(\times\) 10\(^5\) cells), about 5.3% of control. Vehicle (0.2% Me\(_2\)SO) had little effect on the cellular protein kinase C level. This pretreatment of the cells with 1 \(\mu\)M TPA strongly inhibited TPA-induced enhancement of catecholamine secretion from digitonin-permeabilized cells. It did not cause large changes in total cellular catecholamine amounts, Ca\(^{2+}\)-induced catecholamine secretion, and cAMP-induced enhancement of catecholamine secretion. Our experiments on the down-regulation of protein kinase C are in good agreement with the conclusion that protein kinase C is not essential for primary catecholamine secretion.

**DISCUSSION**

Effects of a variety of protein kinase C inhibitors on catecholamine secretion from electropermeabilized bovine adrenal medullary cells have already been reported (8). All protein kinase C inhibitors tested inhibited Ca\(^{2+}\)-evoked catecholamine secretion as well as TPA-induced enhancement of catecholamine secretion from the electropermeabilized cells. The discrepancy between the previous report and our results probably reflects the difference in specificity of the inhibitors tested. Staurosporine and K-252a are nonselective inhibitors of various protein kinases. However, staurosporine and K-252a inhibit protein kinase C with \(K_i\) values of as low as 3 and 25 nM, respectively, in vitro (12, 13). These values are approximately 2 or 3 orders of magnitude lower than those of the protein kinase C inhibitors that were tested previously. From our results it seems likely that staurosporine and K-252a specifically inhibit TPA-dependent regulatory processes of the membrane events (membrane fusion and fission) in exocytosis but not primary membrane event(s) itself.
Ca\textsuperscript{2+}- and Phorbol Ester-dependent Protein Phosphorylation

FIG. 5. Effect of long term treatment of TPA on catecholamine secretion from bovine adrenal medullary cells. 3-day-cultured cells were incubated for 24 h with culture medium containing 0 or 1 \( \mu \text{M} \) TPA. The cells were then washed three times with permeabilizing medium. A, the cells were preincubated for 15 min with permeabilizing medium containing 0 or 100 nm TPA and were then stimulated for 10 min with permeabilizing medium containing 10 \( \mu \text{M} \) digitonin, 2 mM MgATP, 0 or 0.95 \( \mu \text{M} \) Ca\textsuperscript{2+}, and 0 or 100 nM TPA. B, the cells were preincubated for 6 min with permeabilizing medium containing 10 \( \mu \text{M} \) digitonin and 1 mM EGTA and were then stimulated for 10 min with permeabilizing medium containing 2 mM MgATP, 0 or 0.95 \( \mu \text{M} \) Ca\textsuperscript{2+}, and with or without 50 \( \mu \text{M} \) cAMP. In parallel experiments total cellular protein kinase C activity was measured as described under “Experimental Procedures.” Values of protein kinase C activity in the cells pretreated with vehicle (0.2% MeSO) and 1 \( \mu \text{M} \) TPA were 6.45 \( \pm \) 0.22 and 0.34 \( \pm \) 0.06 (nmol of ATP/10 min/S \times 10\textsuperscript{6} cells), respectively. Values shown are means \( \pm \) S.E. of three experiments. A, C, Ca\textsuperscript{2+}, Ca, 0.95 \( \mu \text{M} \) Ca\textsuperscript{2+}.

The effects of staurosporine and K-252a on Ca\textsuperscript{2+}-activated protein kinase C without TPA or diacylglycerol have not been reported. The possibility that Ca\textsuperscript{2+}-activated protein kinase C without TPA might be less sensitive to drug inhibition than is the case with Ca\textsuperscript{2+}-activated protein kinase C with TPA is possible. However, because protein kinase C cannot be activated without TPA or diacylglycerol at physiological concentrations of Ca\textsuperscript{2+} (24), such a possibility cannot account for our results.

Protein kinase C is a family of at least seven subtypes having Ca\textsuperscript{2+}-dependent types (\( \alpha, \beta, \beta', \) and \( \gamma \)) and Ca\textsuperscript{2+}-independent types (\( \delta, \epsilon, \) and \( \zeta \)) (25). At present, the distribution of these protein kinase C subtypes in bovine adrenal medullary cells and their relevance to catecholamine secretion have not been reported. However, Ca\textsuperscript{2+}-independent protein kinase C subtypes should not be a “Ca\textsuperscript{2+} receptor” which is responsible for primary catecholamine secretion. Moreover, because TPA itself cannot induce catecholamine secretion without Ca\textsuperscript{2+}, Ca\textsuperscript{2+}-independent subtypes are not putative intermediate between Ca\textsuperscript{2+} receptor and exocytosis.

K-252a had little effect on Ca\textsuperscript{2+}-evoked catecholamine secretion from digitonin-permeabilized adrenal medullary cells (Figs. 1 and 2). Staurosporine showed only a little inhibition of Ca\textsuperscript{2+}-induced catecholamine secretion. However, these compounds markedly inhibited phosphorylation of many proteins (Figs. 3 and 4). It has been reported that an increase in protein phosphorylation may be essential for initiating catecholamine secretion (3). From our results it seems unlikely that Ca\textsuperscript{2+}-induced phosphorylation of these proteins is a prerequisite for catecholamine secretion. However, further experiments are required to determine exactly the relationship between the initiation of catecholamine secretion and phosphorylation of each protein.

K-252a was reported to inhibit concomitantly serotonin release and phosphorylation of proteins of 20 and 40 kDa without a significant effect on the rise of intracellular free Ca\textsuperscript{2+} induced by platelet-activating factor, suggesting that phosphorylation of these proteins may be a prerequisite for serotonin release in platelets (26). In our work K-252a inhibited phosphorylation of many proteins in digitonin-permeabilized bovine adrenal medullary cells but did not inhibit catecholamine secretion evoked by Ca\textsuperscript{2+} (Figs. 1-4). Although we have no definitive explanation for this discrepancy at present it seems conceivable that it is because of a difference in the regulatory systems of exocytosis. One of the most striking differences of Ca\textsuperscript{2+}-dependent exocytotic release between platelets and adrenal medullary cells is its sensitivity to phorbol ester. TPA has been shown to cause a much larger increase in the apparent affinity of exocytosis for Ca\textsuperscript{2+} in electroporatable platelets than that in electroporatable adrenal medullary cells (27).

We conclude that protein kinase C plays a modulatory role in catecholamine secretion rather than being essential for initiating catecholamine secretion in bovine adrenal medullary cells.

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