Biochimica et Biophysica Acta, 979 (1989) 157–165
Elsevier

BBA 74311

Interaction of protein kinase C with chromaffin granule membranes: effects of Ca^{2+}, phorbol esters and temperature reveal differences in the properties of the association and dissociation events

Keith W. Brocklehurst and Harvey B. Pollard

Laboratory of Cell Biology and Genetics, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD (U.S.A.)

(Received 1 August 1988)

Key words: Adrenal medulla; Exocytosis; Stimulus secretion; Chromaffin granule; Protein phosphorylation; Protein kinase C

Interaction of protein kinase C with chromaffin granule membranes has been studied as a means of investigating the translocation of protein kinase C from cytosol to intracellular membrane surfaces, which is believed to occur during secretion. Protein kinase C in an adrenal medullary soluble fraction was found to bind reversibly to granule membranes in a Ca^{2+}-dependent fashion. Association and dissociation events were sensitive to Ca^{2+} concentrations in the low micromolar range, and the Ca^{2+} sensitivity of both processes was increased when the membranes had been preincubated with the protein kinase C-activating phorbol ester, 4β-phorbol 12-myristate 13-acetate (TPA). Binding of protein kinase C to granule membranes occurred at 0 and 37°C, irrespective of whether the membranes had been preincubated with TPA. However, dissociation of protein kinase C from granule membranes that had been preincubated with TPA occurred only at 37°C and not at 0°C, even though dissociation of the enzyme from membranes which had not been preincubated with TPA would occur at both 37 and 0°C. These effects of TPA were not reproduced by 4α-phorbol 12,13-didecanoate (4αPDD), a phorbol ester which does not activate protein kinase C. Soluble protein kinase C activity also associated with chromaffin granules in a Ca^{2+}-dependent manner in an adrenal medullary homogenate, indicating that granules can compete with other intracellular membranes for the binding of protein kinase C. Results obtained with this model system differ from other systems where the interaction of protein kinase C with plasma membranes has been studied and have general implications for studies performed on the translocation of protein kinase C in intact cells and for the role of protein kinase C in stimulus-secretion coupling in the chromaffin cell.

Introduction

There is compelling evidence that protein kinase C, a Ca^{2+}-activated phospholipid-dependent protein kinase, is involved in signal transduction in secretory and other cell types [1]. It has been proposed that the second messenger for protein kinase C activation is diacylglycerol, a product of the receptor-mediated turnover of polyphosphoinositides at the plasma membrane, since diacylglycerol increases the affinity of the enzyme for both Ca^{2+} and phospholipid [1]. The phorbol ester, TPA, has also been shown to activate protein kinase C in a manner analagous to diacylglycerol [2] and it appears that the cellular receptor for TPA is protein kinase C [3]. It has become clear recently that there are several subspecies of protein kinase C that have subtle variations in biochemical properties and different tissue distributions [1].

Activation of protein kinase C in many cell types is associated with translocation of the enzyme from the cytosol to a membrane fraction. The stimulus-induced translocation of protein kinase C is thought to be occasioned by the production of diacylglycerol in the plasma membrane and this translocation event can also be induced by TPA [4–10]. In the bovine adrenal medullary chromaffin cell, however, activation of the secretory mechanism by the physiological secretagogue, acetylcholine, appears to be primarily mediated by

Abbreviations: TPA, 4β-phorbol 12-myristate 13-acetate; 4αPDD, 4α-phorbol 12,13-didecanoate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Correspondence: K.W. Brocklehurst, Laboratory of Cell Biology and Genetics, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

nicotinic cholinergic receptors, whereas the turnover of polyphosphoinositides is mediated by muscarinic cholinergic receptors [11,12]. Thus, if protein kinase C plays a role in nicotine-induced catecholamine secretion from bovine chromaffin cells, it is unlikely to be mediated by an increased diacylglycerol content of the plasma membrane, but may instead be mediated by the rise in cytosolic Ca^{2+} concentration brought about by nicotine-induced Ca^{2+} influx [13]. If this is the case, then intracellular membranes other than the plasma membrane may be targets for the stimulus-induced translocation of protein kinase C and, given the large surface area of chromaffin granule membrane inside the cell, these membranes are an obvious potential target. Indeed it has previously been shown that protein kinase C will associate with isolated chromaffin granule membranes [14].

It was the aim of this investigation to study further the interaction of protein kinase C with chromaffin granules in an attempt to model in vitro the events associated with protein kinase C activation in the chromaffin cell. For these studies, the source of protein kinase C used was a soluble fraction prepared from bovine adrenal medulla, previously shown to contain high levels of protein kinase C activity [15–17], which has been fully characterized [15,16]. This fraction was chosen as the source of protein kinase C, rather than the purified enzyme, because the high levels of protein kinase C present in the bovine adrenal medulla compared to other tissues makes it possible to study the interaction of the enzyme with chromaffin granules in the context of a protein environment reminiscent of that found inside the cell.

Materials and Methods

Preparation of subcellular fractions

Chromaffin granule membranes were prepared at 4°C by a modified version of the method of Bartlett and Smith [18]. Medullae from four fresh bovine adrenal glands were disrupted for 6 s in a Waring blender and then homogenized with a loose-fitting Teflon pestle in a glass homogenizer in 40 ml of homogenization buffer containing 10 mM Mes-Tris (pH 6.5)/0.3 M sucrose/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through surgical gauze and the filtrate was centrifuged at 2000 rpm for 10 min in a Sorvall SS-34 rotor. The supernatant was then centrifuged at 13000 rpm for 30 min in the SS-34 rotor and the resulting pellet was resuspended in 2 ml of homogenization buffer. This suspension was layered on top of 6 ml of homogenization buffer containing 1.6 M – instead of 0.3 M – sucrose in a Beckman type 40 rotor tube and centrifuged at 40000 rpm for 50 min. The granule pellet was lysed in 40 ml of regular homogenization buffer and centrifuged at 20000 rpm for 15 min in the SS-34 rotor. The pellet was then resuspended in 40 ml of 0.3 M sucrose and again centrifuged at 20000 rpm for 15 min. The pellet was this time resuspended in 0.5 ml of 0.3 M sucrose, and centrifuged for 10 min in an Eppendorf microfuge. The pellet from this centrifugation was finally resuspended in 0.25 ml of 0.3 M sucrose. The protein concentration of the granule membrane preparation was determined [19] using bovine serum albumin (Calbiochem-Behring, San Diego, CA) as standard. The preparation was then diluted with 0.3 M sucrose to give a protein concentration of 25 mg/ml.

In the preparation of an adrenal medullary soluble fraction, medullae from ten fresh adrenal glands were processed as described above, except that after centrifugation of the homogenate at 13000 rpm the supernatant was taken and further centrifuged at 50000 rpm for 30 min in a Beckman SW60 rotor. The yellow lipid film found floating on top of the tubes was removed with tissue paper and the remaining supernatant concentrated approx. 5-fold using Centricon 10 microconcentrators (Amicon, Danvers, MA). Typically, the concentrated fraction had a protein concentration of between 50 and 85 mg/ml.

General protocol for the measurement of the reversible association of protein kinase C with chromaffin granule membranes

Aliquots (20 μl) of granule membranes were preincubated for 10 min at 37°C with 80 μl of a solution of 0.3 M sucrose containing, when required, an appropriate concentration of phorbol ester (obtained from Sigma and stored as 1 mM stock solutions in dimethylsulfoxide. Control preincubations contained a corresponding amount of dimethylsulfoxide alone). The membranes were isolated by centrifugation for 10 min at 4°C in an Eppendorf microfuge. The pellets were resuspended in 50 μl of a solution containing 5 μl of adrenal medullary soluble fraction and 50 mM Mes-Tris (pH 6.7)/0.27 M sucrose/1 mM $MgCl_2$/0.1 mM dithiothreitol/5 mM EGTA and various concentrations of $CaCl_2$ (free Ca^{2+} concentrations were calculated as previously described [15]) and incubated for 10 min at 37°C. The membranes were again isolated by centrifugation and in experiments performed to measure binding the supernatants were assayed for removal of protein kinase C activity.

In experiments designed to measure the subsequent dissociation of bound protein kinase C from membranes, the pellets from the last centrifugation were resuspended in 50 μl of a solution containing 50 mM Mes-Tris (pH 6.7)/0.3 M sucrose/1 mM $MgCl_2$/0.1 mM dithiothreitol/5 mM EGTA and various concentrations of $CaCl_2$. After incubation for 10 min at 37°C the membranes were again isolated by centrifugation and the supernatant assayed for released protein kinase C activity.

Incubation times of 10 min were used for both binding and release experiments as steady-state conditions were achieved by this time (data not shown).

Comparison of the binding of protein kinase C to chromaffin granules in an adrenal medullary homogenate and in a reconstituted system of granule membranes and a medullary soluble fraction

Medullae from ten fresh adrenal glands were disrupted in 40 ml of homogenization buffer and a 2000 rpm supernatant prepared as described above. Aliquots (2 ml, corresponding to approx. 25 mg of protein) of this supernatant were then incubated at 37°C for 10 min in a final vol. of 20 ml of a solution containing in addition 50 mM Mes-Tris (pH 6.7)/0.27 M sucrose/1 mM $MgCl_2$/5 mM EGTA, either in the absence or presence of 5 mM $CaCl_2$. The granules from each incubation mixture were then isolated at 4°C either in the absence or presence of $CaCl_2$. The incubation mixtures were centrifuged at 13000 rpm for 30 min and the resulting pellets were suspended in 2.5 ml of resuspension buffer, consisting of 50 mM Mes-Tris (pH 6.7)/0.3 M sucrose/1 mM $MgCl_2$/5 mM EGTA, either in the absence or presence of 5 mM $CaCl_2$. These suspensions were then layered on top of 6 ml of the same respective solutions except that the sucrose concentrations were 1.6 M instead of 0.3 M. After centrifugation at 40000 rpm for 50 min, the granule pellets were lysed in 20 ml of regular resuspension buffer, again either in the absence or presence of 5 mM $CaCl_2$. The suspensions were then centrifuged at 20000 rpm for 15 min and the pellets were resuspended in 0.5 ml of resuspension buffer minus or plus 5 mM $CaCl_2$. After further centrifugation for 10 min in a microfuge, both pellets were resuspended in 0.15 ml of resuspension buffer in the absence of $CaCl_2$ and incubated for 10 min at 37°C. The membranes were finally separated by centrifugation for 10 min in the microfuge and the resulting supernatants were assayed for released protein kinase C activity.

Concurrently with the above procedure, two more aliquots (2 ml) of 2000 rpm medullary supernatant were used to prepare granule membranes. To each aliquot was added 18 ml of homogenization buffer and the suspensions were centrifuged at 13000 rpm for 30 min. The pellets were resuspended in 2.5 ml of homogenization buffer and layered on top of 6 ml of homogenization buffer containing 1.6 M sucrose. After centrifugation at 40000 rpm for 50 min, the pellets were resuspended in 20 ml of regular homogenization buffer and again centrifuged at 20000 rpm for 15 min. The pellets were then resuspended and incubated for 10 min at 37°C in 20 ml of a solution containing 1.76 ml of adrenal medullary soluble fraction (prepared as described below from the same homogenate) and 50 mM Mes-Tris (pH 6.7)/0.27 M sucrose/1 mM $MgCl_2$/5 mM EGTA, in the absence or presence of 5 mM $CaCl_2$. The membranes were separated by centrifugation at 20000 rpm for 15 min and the pellets were resuspended in 0.5 ml of resuspension buffer in the absence or presence of 5 mM $CaCl_2$. After centrifugation for 10 min in the microfuge, both pellets were resuspended in 0.15 ml of resuspension buffer in the absence of $CaCl_2$ and incubated at 37°C for 10 min. The membranes

TABLE I

Effect of temperature and TPA-pretreatment of chromaffin granule membranes on (a) the binding of protein kinase C and on (b) the dissociation of bound protein kinase C

Chromaffin granule membranes were preincubated in the absence or presence of a final concentration of 10^{-7} M TPA and the membranes separated by centrifugation. The membranes were then treated as follows. (a) The binding of protein kinase C was measured in the absence or presence of 5 mM $CaCl_2$ either at 0 or 37°C. Results were calculated as a percentage of the maximum activity remaining in the supernatant for each experiment and are shown as mean ± S.E. for three separate experiments. (b) The pellets were incubated with adrenal medullary soluble fraction in the absence or presence of 5 mM $CaCl_2$ and either at 0 or 37°C and the membranes were again isolated by centrifugation. The pellets were then resuspended in the absence of $CaCl_2$ at 0 or 37°C and the released protein kinase C activity measured. Results were calculated as a percentage of the maximum activity released in each experiment and are shown as mean ± S.E. for three separate experiments.

(a) Binding

Granule membranes pretreated with TPA	$CaCl_2$ present during binding incubation	Binding temperature (°C)	Protein kinase C activity remaining in supernatant (% of maximum)
–	–	0	85 ± 10
–	–	37	89 ± 6
–	+	0	15 ± 0
–	+	37	0 ± 0
+	–	0	84 ± 10
+	–	37	81 ± 1
+	+	0	0 ± 0
+	+	37	0 ± 0

(b) Dissociation

Granule membranes pretreated with TPA	$CaCl_2$ present during binding incubation	Binding/elution temperature (°C)	Protein kinase C activity released into supernatant (% of maximum)
–	–	0/0	7 ± 4
–	–	37/37	6 ± 2
–	+	0/0	79 ± 8
–	+	0/37	100 ± 0
–	+	37/0	64 ± 8
–	+	37/37	79 ± 3
+	–	0/0	9 ± 2
+	–	37/37	16 ± 8
+	+	0/0	3 ± 1
+	+	0/37	78 ± 4
+	+	37/0	4 ± 1
+	+	37/37	74 ± 4

were finally isolated by centrifugation for 10 min in the microfuge and the supernatants were assayed for released protein kinase C activity.

The adrenal medullary soluble fraction used in the reconstitution experiment was prepared from the remainder of the 2000 rpm supernatant as described in the previous section. The volumes of the 2000 rpm supernatant and final soluble fraction were measured in order to calculate the volume of soluble fraction equivalent to 2 ml of 2000 rpm supernatant, and this value was found to be 1.76 ml (typically equivalent to approx. 10–15 mg of protein).

The protein content of the final four granule membrane pellets in the above experiment were measured and the results presented in Table III are corrected for the slightly different yields of membrane protein obtained under the different conditions (typically, the recovered membrane protein was approx. 2–3 mg).

Protein kinase C assay

Samples for assay were diluted 10-fold with H_2O to reduce their EGTA/$CaCl_2$ concentrations. Aliquots (10 μl) were then assayed in a final reaction vol. of 50 μl as previously described [15], with the exception that the EGTA and $CaCl_2$ concentrations contributed by the reaction mixture were 0.5 and 1 mM, respectively. Samples were assayed in the presence of 40 μg of phosphatidylserine/ml and in the absence or presence of $CaCl_2$.

The protein kinase activity in the absence of $CaCl_2$ was subtracted from that in the presence of $CaCl_2$ to give the protein kinase C activity. None of the samples assayed showed Ca^{2+}-dependent histone kinase activity in the absence of phosphatidylserine, and since control incubations in the presence of phosphatidylserine of sample or histone alone in the assay did not show Ca^{2+}-dependent incorporation of radioactivity into trichloroacetic-acid-precipitable material, it was not necessary to correct for them to obtain the protein kinase C activity of the sample.

Results

Adrenal medullary soluble fraction was incubated with increasing concentrations of chromaffin granule membranes for 10 min at 37°C in the presence of 30 μM free Ca^{2+} and the membranes then isolated by centrifugation. It was found that, as the membrane concentration was increased, the amount of protein kinase C activity remaining in the supernatant decreased until no soluble activity remained (Fig. 1). For all subsequent binding experiments an excess of membrane protein was routinely used to ensure complete binding of protein kinase C activity under optimal conditions.

The removal of protein kinase C activity from the adrenal medullary soluble fraction by granule membranes was Ca^{2+} dependent as illustrated in Fig. 2a, with half-maximal removal of activity being apparent at a free Ca^{2+} concentration of approx. 7 μM. However, if the membranes had been preincubated with 10^{-7} M TPA before incubation with the medullary soluble fraction, then the Ca^{2+} concentration dependence of the removal of protein kinase C activity was shifted to the left, such that half-maximal removal now occurred at approx. 1 μM free Ca^{2+}.

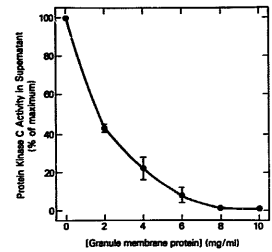

Fig. 1. Effect of chromaffin granule membrane protein concentration on the binding of protein kinase C. Aliquots (0–20 μl) of chromaffin granule membranes were made up to a final vol. of 0.1 ml with 0.3 M sucrose and centrifuged for 10 min at 4°C in an Eppendorf microfuge. The binding of protein kinase C present in an adrenal medullary soluble fraction to the membranes in the presence of 5 mM $CaCl_2$ (free Ca^{2+} concentration of approx. 30 μM) was then measured as described in Materials and Methods. Results were calculated as a percentage of the maximum activity observed remaining in the supernatant in each experiment (which was approx. 80 pmol phosphate transferred to histone/min in each case) and are shown as the means ± range for two experiments. The final protein concentration of the soluble fraction in the incubation mixtures in these experiments was 4.9 mg/ml.

The Ca^{2+}-dependent binding of protein kinase C to granule membranes was found to be reversible upon removing Ca^{2+} from the incubation medium. Membranes which had been incubated with the medullary soluble fraction in the presence of 30 μM free Ca^{2+}, separated by centrifugation and then resuspended in the absence of Ca^{2+}, released 65 ± 9% (mean ± S.E.; $n = 4$) of the activity that was removed from the medullary soluble fraction (no activity was released under these conditions if the membranes had not been preincubated with the soluble fraction). It is of interest that the results of preliminary experiments demonstrate a similar Ca^{2+}-dependent binding of protein kinase C present in the adrenal medullary soluble fraction to phosphatidylserine liposomes, but only a small percentage (3–24%) of the bound activity was eluted from the liposomes upon removing Ca^{2+} from the medium.

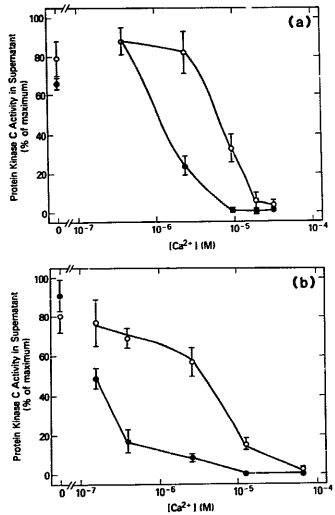

Fig. 2. Effect of TPA pretreatment of chromaffin granule membranes on the Ca^{2+} concentration dependency of (a) the binding of protein kinase C and on (B) the dissociation of bound protein kinase C. Chromaffin granule membranes were preincubated either with (●) or without (○) 10^{-7} M TPA. After separation by centrifugation the membranes were then treated as follows. (a) The binding of protein kinase C was measured at various concentrations of $CaCl_2$. Results were calculated as a percentage of the maximum activity observed remaining in the supernatant in each experiment and are shown as the mean ± S.E. for four separate experiments. (b) Protein kinase C was allowed to bind to the membranes by incubating with adrenal medullary soluble fraction in the presence of 5 mM $CaCl_2$. After separation of the membranes by centrifugation, they were resuspended in the presence of various concentrations of $CaCl_2$ and the released protein kinase C activity measured. Results were calculated as a percentage of the maximum activity observed released into the supernatant in each experiment and are shown as the mean ± S.E. for five separate experiments.

On examination of the Ca^{2+} dependency of the release of protein kinase C from chromaffin granule membranes, progressively less activity was released as the Ca^{2+} concentration of the medium was increased (Fig. 2b). Half-maximal release of enzyme activity was achieved at approx. 5 μM free Ca^{2+}. However, if the membranes had been preincubated with 10^{-7} M TPA before incubation with the adrenal medullary soluble fraction, then the Ca^{2+} concentration dependency of the subsequent release of protein kinase C activity from the membranes was reduced so that half-maximal release was now achieved at approx. 0.2 μM.

The effect of TPA concentration during the preincubation of granule membranes on the subsequent binding of protein kinase C is shown in Fig. 3a. For this experiment a free Ca^{2+} concentration of approx. 2 μM was chosen at which to measure binding to best illustrate the effect of TPA. As the TPA concentration of the preincubation medium was increased, there was a progressive increase in the amount of protein kinase C activity which bound to membranes. Similarly, Fig. 3b illustrates the effect of TPA concentration during the preincubation with granule membranes on the dissocia-

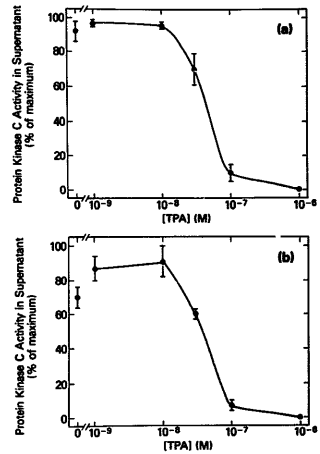

Fig. 3. Effect of TPA concentration during pretreatment of chromaffin granule membranes on the binding of protein kinase C and on the dissociation of bound protein kinase C. Chromaffin granule membranes were preincubated in the presence of various concentrations of TPA. After separation by centrifugation the membranes were then treated as follows. (a) The binding of protein kinase C was measured in the presence of 3.8 mM $CaCl_2$ (free Ca^{2+} concentration of approx. 2 μM). Results were calculated as a percentage of the maximum activity observed remaining in the supernatant in each experiment and are shown as the mean ± S.E. for three separate experiments. (b) Protein kinase C was allowed to bind to the membranes by incubating with adrenal medullary soluble fraction in the presence of 5 mM $CaCl_2$. After separation of the membranes by centrifugation, they were resuspended in the presence of 3.8 mM $CaCl_2$ (free Ca^{2+} concentration of approx. 3 μM) and the released protein kinase C activity measured. Results were calculated as a percentage of the maximum activity observed released into the supernatant in each experiment and are shown as mean ± S.E. for four separate experiments.

tion of protein kinase C activity from membranes. Again for this experiment a free Ca^{2+} concentration of the dissociation medium of approx. 3 μM was chosen that would make readily apparent the effect of TPA. The two TPA concentration dependencies shown in Fig. 3 are very similar to half-maximal effects seen at around 50 nM.

To investigate the temperature dependence of binding of protein kinase C to chromaffin granule membranes the extent of the binding reaction at an optimal free Ca^{2+} concentration was compared at 0°C and 37°C (Table Ia). Virtually no difference was found between the extent of binding observed at the two temperatures and there was no difference in this respect between membranes that had been preincubated with TPA and those which had not. Similarly, the effect of temperature on the dissociation of protein kinase C from granule membranes is shown in Table I(b). It was found that for membranes which had not been preincubated with TPA, dissociation of protein kinase C in the absence of added Ca^{2+} occurred equally well at 0°C and 37°C. However, it was of great interest to discover that, for the membranes that had been preincubated with TPA, the subsequent elution of protein kinase C was apparent only at 37°C and did not take place at 0°C, irrespective of whether the prior binding reaction had been conducted at 0°C or 37°C.

The effects of TPA on the interaction of protein kinase C with chromaffin granule membranes were compared with those of the phorbol ester, 4αPDD, which is incapable of activating protein kinase C [2]. Preincubation of chromaffin granule membranes with 10^{-7} M TPA resulted in virtually the complete binding of protein kinase C upon incubation with adrenal medullary soluble fraction at a free Ca^{2+} concentration of approx. 2 μM. However, the binding observed after preincubation with an equivalent concentration of 4αPDD was slight and equivalent to that seen with untreated membranes (Table II (a)). Similarly, whilst membranes that had been preincubated with TPA did not release bound protein kinase C activity in the absence of Ca^{2+} at 0°C, membranes which had been preincubated with 4αPDD released an equivalent amount of protein kinase C activity to that released from untreated membranes (Table IIb).

In order to determine whether soluble protein kinase C present in an adrenal medullary homogenate would become associated with chromaffin granules in the presence of Ca^{2+}, given the presence of other possible competing membrane surfaces, the following experiment was undertaken. Aliquots of a post-nuclear homogenate were incubated with or without Ca^{2+} and the granules present in the homogenate were then respectively prepared as membranes with or without Ca^{2+} present during the isolation procedure. The membranes from both preparations were finally incubated in the

TABLE II

Comparison of the effects of TPA- and 4αPDD-pretreatment of chromaffin granule membranes on (a) the binding of protein kinase C and on (b) the dissociation of bound protein kinase C at 0°C

Chromaffin granule membranes were preincubated in the absence of phorbol ester or in the presence of a final concentration of 10^{-7} M TPA or 4αPDD and the membranes were then isolated by centrifugation. The pellets were then treated as follows. (a) The binding of protein kinase C was measured in the presence of either (i) 0 mM-, (ii) 3.8 mM- (free Ca^{2+} concentration of approx. 2 μM) or (iii) 5 mM $CaCl_2$ (free Ca^{2+} concentration of approx. 30 μM). Results were calculated as a percentage of the maximum activity remaining in the supernatant for each experiment and are shown as the mean ± S.E. for three separate experiments. (b) Protein kinase C was allowed to bind to the membranes by incubating with adrenal medullary soluble fraction in the presence of 5 mM $CaCl_2$ at 37°C. After separation of the membranes by centrifugation, they were resuspended in the absence of $CaCl_2$ and incubated at 0°C. Released protein kinase C was measured. Results were calculated as a percentage of the maximum activity released in each experiment and are shown as the mean ± S.E. for three separate experiments.

(a) Binding

Pretreatment of granule membranes	[$CaCl_2$] of incubation mixture (mM)	Protein kinase C activity remaining in supernatant (% of maximum)
–	0	97 ± 2
–	3.8	82 ± 8
–	5.0	0 ± 0
TPA	0	93 ± 4
TPA	3.8	4 ± 1
TPA	5.0	0 ± 0
4αPDD	0	97 ± 3
4αPDD	3.8	78 ± 5
4αPDD	5.0	0 ± 0

(b) Dissociation

Pretreatment of granule membranes	Protein kinase C activity released into supernatant (% of maximum)
–	84 ± 11
TPA	2 ± 1
4αPDD	95 ± 5

absence of Ca^{2+} and the supernatants of these incubations assayed for released protein kinase C activity (Table III). Granule membranes isolated from the homogenate that had been incubated with Ca^{2+} were found to release protein kinase C activity under these conditions, whereas the membranes isolated from the homogenate that had been incubated without Ca^{2+} did not. Also show in Table III are the results of a parallel experiments in which the granule membranes and soluble fraction prepared from an equal volume of post-nuclear homogenate were reconstituted together without competing membranes and the binding of protein kinase C to membranes was studied under identical conditions. Again, as would be expected, Ca^{2+} was required for the association of protein kinase C activity with the granule membranes. It can also be seen that, in the homogenate

TABLE III

Comparison of the effects of Ca^{2+} on the binding of protein kinase C to chromaffin granules in an adrenal medullary homogenate and in a reconstituted system of chromaffin granule membranes and an adrenal medullary soluble fraction

For details see Materials and Methods. In each case, results are expressed as a percentage of the total initial soluble protein kinase C activity present in the binding incubations (which varied from 5000 to 7000 pmol/min for the different experiments) and are shown as the mean ± S.E. for three separate experiments.

Binding conditions	Protein kinase C activity eluted from membranes (% of total initial soluble activity)
(a) Homogenate	
$-CaCl_2$	0.2 ± 0.1
$+CaCl_2$	7.2 ± 1.2
(b) Membranes + soluble fraction	
$-CaCl_2$	0.2 ± 0.0
$+CaCl_2$	16.5 ± 3.4

incubation, the amount of protein kinase C activity that associated with granules was approx. 45% of the amount of activity that associated with the granules in the reconstituted system, which is devoid of competing membranes.

Discussion

The experiments reported here were undertaken in order to characterize the interaction of protein kinase C with chromaffin granules in an attempt to model in vitro the events associated with protein kinase C activation in the chromaffin cell. It was thus found that protein kinase C present in the adrenal medullary soluble fraction binds reversibly to chromaffin granule membranes in a Ca^{2+}-dependent manner. Furthermore, TPA was found to increase the Ca^{2+} sensitivity of both the binding and elution events in a concentration-dependent fashion. This effect of TPA was not reproduced by 4αPDD. The range of Ca^{2+} concentrations effective in promoting the binding of protein kinase C to granule membranes is consistent with the levels of cytosolic Ca^{2+} achieved on stimulation of chromaffin cells with acetylcholine [13]. Although the Ca^{2+} sensitivities of both binding and dissociation of protein kinase C from chromaffin granule membranes is approximately the same in the absence of TPA pretreatment, for membranes which have been preincubated with TPA, the Ca^{2+} sensitivity of protein kinase C dissociation from the membranes is greater than the Ca^{2+} sensitivity of binding of the enzyme to the membranes.

The binding of protein kinase C to chromaffin granule membranes was found to occur both at 0°C and 37°C, irrespective of whether the membranes had been pretreated with TPA. However, it was of great interest to discover that, whilst the dissociation of protein kinase C from membranes which had not been pretreated with TPA occurred both at 0°C and 37°C, the dissociation of enzyme activity from membranes which had been pretreated with TPA would only occur at 37°C and not at 0°C. The mechanism whereby TPA mediates this effect is not known, but may reflect a profound effect of TPA on membrane structure. Again this effect of TPA was not reproduced by 4αPDD.

In the intact chromaffin cell there are many membrane surfaces other than the chromaffin granule membrane which may compete for the binding of cytosolic protein kinase C. However, it was observed that soluble protein kinase C would still associate with chromaffin granules to a significant degree in an adrenal medullary homogenate in a Ca^{2+}-dependent manner. The extent of association was found to be approx. 45% of that observed when corresponding amounts of soluble fraction and chromaffin granule membranes to those present in the homogenate were reconstituted together. Thus, it appears that chromaffin granules can effectively compete with other membrane surfaces in the cell for the binding of protein kinase C. It should be remembered, though, that this experiment does not take into account binding to nuclei, which are removed along with cell debris before incubation, nor binding to the inner leaflet of the plasma membrane, since plasma membrane vesicles in the homogenate are likely to be oriented outside-out.

The results obtained from this in vitro model have two important general implications for studies on the intracellular translocation and activation of protein kinase C in intact cells. Firstly, complexes formed between protein kinase C and membranes solely as a result of an increase in the cytosolic Ca^{2+} concentration are unlikely to be detected after homogenization of cells with Ca^{2+} chelators. And secondly, in order to demonstrate the intracellular translocation of protein kinase C from cytosol to membrane induced by TPA or perhaps the receptor-mediated production of diacylglycerol, it may be essential to homogenize the cells on ice; fortunately, this appears to have been the standard technique for this type of experiment. Examples of the first of these two points are already apparent. Thus, in GH_3 pituitary cells, thyrotropin-releasing hormone, which promotes phosphoinositide turnover in these cells, phospholipase C, a permeable diacylglycerol and TPA all induced a cytosol to membrane translocation of protein kinase C [4]. This effect was not seen; however, when the cells were stimulated with K^+ or Ba^{2+}. Moreover, in the bovine chromaffin cell, TPA has been shown to cause a substantial translocation of protein kinase C from cytosol to membrane, but a nicotinic agonist had a much smaller effect [20]. In the same study, the leakage of protein kinase C from digitonin-permeabilized chro-

maffin cells was investigated. It was found that Ca^{2+} caused retention of protein kinase C activity in the permeabilized cells, the enzyme presumably having become associated with intracellular membranes, but, after subsequent homogenization in the presence of EGTA, most of the protein kinase C activity remaining in the cells was found to be soluble.

Other model systems have been developed for studying the interaction of protein kinase C with membranes. For example, Wolf et al. [21,22] used inside-out human erythrocyte vesicles and protein kinase C purified from rat brain. They also showed that phorbol ester, in this case phorbol 12,13-dibutyrate, increased the Ca^{2+} sensitivity of both the binding of protein kinase C to the membranes and its subsequent dissociation. However, in the erythrocyte membrane system, in the presence of the phorbol ester incomplete dissociation of the enzyme from the membranes occurred upon removing Ca^{2+} from the medium, and complete release of enzyme activity was achieved only in the presence of ATP. Since neither the amount of protein kinase C activity which binds to chromaffin granule membranes at saturating Ca^{2+} concentrations, nor the amount of activity which subsequently dissociates from the granule membranes in the presence of EGTA, is affected by TPA, it thus appears that chromaffin granule membranes may have properties different from plasma membranes with respect to interactions with protein kinase C.

In another system the binding of partially purified bovine brain protein kinase C to parietal yolk sac membranes was found not to occur in the presence of Ca^{2+} and TPA unless exogenous phosphatidylserine was added [23]. The authors of this study distinguished two types of interaction of protein kinase C with membranes: a superficial, reversible, Ca^{2+}-dependent binding and a more stable binding, seen in the presence of TPA, which was still apparent in the presence of chelator. The latter type of association was observed in subcellular fractions enriched in plasma membranes and nuclei, but not in fractions containing mitochondria or microsomes. The TPA-induced binding was also slower at 4°C than at 25°C, whereas Ca^{2+}-induced binding was temperature independent.

Thus, the properties exhibited by these other two model systems indicate that there may even be differences between plasma membranes from different cell types in the nature of their interactions with protein kinase C. The relative irreversibility of the Ca^{2+}-dependent association of protein kinase C with phosphatidylserine liposomes reported in this communication also indicates that the association of protein kinase C with chromaffin granules is not merely mediated by phosphatidylserine present in the granule membrane.

The results reported here also offer insight into the role of protein kinase C in stimulus-secretion coupling in the chromaffin cell. The effects of TPA on protein phosphorylation in intact chromaffin cells occur in the absence of extracellular Ca^{2+} [24], and this is also made apparent by the fact that TPA-induced activation of tyrosine hydroxylase can occur in the absence of extracellular Ca^{2+} [25,26]. However, the effects of TPA on catecholamine secretion from intact cells require the presence of extracellular Ca^{2+} and are most marked in the presence of an increase in the cytosolic Ca^{2+} concentration [15,24,27,28]. Similarly, the effects of TPA on catecholamine release from permeabilized chromaffin cells are Ca^{2+} dependent [24,29,30]. These results imply that the translocation and activation of protein kinase C induced by TPA in chromaffin cells can occur at the resting cytosolic Ca^{2+} concentration even in the absence of extracellular Ca^{2+}, and that a separate Ca^{2+}-dependent step is needed to allow exocytosis to occur. The resting cytosolic Ca^{2+} concentration in chromaffin cells is normally of the order of 0.1 μM [13] and is probably even lower in the absence of extracellular Ca^{2+}, whereas in the model system described here the binding of protein kinase C to granule membranes in the presence of TPA was half-maximal at around 1 μM Ca^{2+}. This discrepancy may possibly be accounted for by the presence of Ca^{2+} bound to negatively charged groups on the intracellular surface of granule membranes in situ, which may be sufficient to allow the binding of protein kinase C in the presence of TPA. However, a translocation of protein kinase C from the cytosol to granule membranes during nicotine-induced secretion would require the increase in cytosolic Ca^{2+} concentration produced by this secretagogue.

Evidence that an intracellular translocation of protein kinase C from cytosol to secretory granules may be important in the process of exocytosis has been provided by the demonstration of the existence of protein kinase C substrates associated with insulin-secretory granules [31,32], adenohypophyseal luteinizing hormone secretory granules [33] and pancreatic zymogen granules [34]. Association of protein kinase C with secretory granule membranes may however also serve to activate the enzyme towards cytosolic substrates, as appears to be the case for the 37000 M_r substrate CB9 in the chromaffin cell [14,35]. It is also worthy to note that a phosphatidylinositol-specific phospholipase C present in the soluble fraction of bovine adrenal medulla has been shown to bind to chromaffin granule membranes in a Ca^{2+}-dependent manner [36]. Association of this enzyme with chromaffin granules mediated by an increase in the cytosolic Ca^{2+} concentration may therefore result in the production of diacylglycerol in the granule membrane, which may further regulate the association of protein kinase C with the granule.

Thus, the model system described here for investigating the events associated with protein kinase C activation during stimulus-secretion coupling has yielded results of relevance to the interpretation of studies on the

intracellular translocation of the enzyme in intact cells. In particular, the observation that TPA differentially affects the binding and dissociation of protein kinase C from granule membranes may be an important clue to the effects of TPA on membrane structure. Further investigations into the substrates phosphorylated as a result of the binding of protein kinase C to chromaffin granules and other possible mechanisms, such as proteolysis, involved in the regulation of this interaction may provide more insight into the mechanism of exocytosis.

Acknowledgements

We would like to thank Dr. J.C. Hutton for computing the free Ca^{2+} concentrations.

References

1 Nishizuka, Y. (1988) Nature 334, 661–665.
2 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
3 Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953–959.
4 Drust, D.S. and Martin, T.F.J. (1985) Biochem. Biophys. Res. Commun. 128, 531–537.
5 Farrar, W.L. and Anderson, W.B. (1985) Nature 315, 233–235.
6 Farrar, W.L., Thomas, T.P. and Anderson, W.B. (1985) Nature 315, 235–237.
7 Sugden, D., Vanecek, J., Klein, D.C., Thomas, T.P. and Anderson, W.B. (1985) Nature 314, 359–361.
8 Tanabe, A., Nielsen, T.B., Rani, C.S.A. and Field, J.B. (1985) Arch. Biochem. Biophys. 243, 92–99.
9 White, J.R., Pluznik, D.H., Ishizaka, K. and Ishizaka, T. (1985) Proc. Natl. Acad. Sci. USA 82, 8193–8197.
10 Chen, Z.Z., Coggeshall, K.M. and Cambier, J.C. (1986) J. Immunol. 136, 2300–2304.
11 Fisher, S.K., Holz, R.W. and Agranoff, B.W. (1981) J. Neurochem. 37, 491–497.
12 Forsberg, E.J., Rojas, E. and Pollard, H.B. (1986) J. Biol. Chem. 261, 4915–4920.
13 Kao, L.-S. and Schneider, A.S. (1986) J. Biol. Chem. 261, 4881–4888.
14 Summers, T.A. and Creutz, C.E. (1985) J. Biol. Chem. 260, 2437–2443.
15 Brocklehurst, K.W., Morita, K. and Pollard, H.B. (1985) Biochem. J. 228, 35–42.
16 Brocklehurst, K.W., Lee, G. and Pollard, H.B. (1986) Bioscience Rep. 6, 749–757.
17 Wise, B.C. and Costa, E. (1985) J. Neurochem. 45, 227–234.
18 Bartlett, S.F. and Smith, A.D. (1974) Methods Enzymol. 31, 379–389.
19 Bradford, M. (1976) Anal. Biochem. 72, 248–254.
20 TerBush, D.R. and Holz, R.W. (1986) J. Biol. Chem. 261, 17099–17106.
21 Wolf, M., Cuatrecasas, P. and Sahyoun, N. (1985) J. Biol. Chem. 260, 15718–15722.
22 Wolf, M., Levine, H., III, May, W.S., Jr., Cuatrecasas, P. and Sahyoun, N. (1985) Nature 317, 546–549.
23 Gopalakrishna, R., Barsky, S.H., Thomas, T.P. and Anderson, W.B. (1986) J. Biol. Chem. 261, 16438–16445.
24 Pocotte, S.L., Frye, R.A., Senter, R.A., TerBush, D.R., Lee, S.A. and Holz, R.W. (1985) Proc. Natl. Acad. Sci. USA 82, 930–934.
25 Houchi, H., Nakanishi, A., Uddin, M.M., Ohuchi, T. and Oka, M. (1985) FEBS Lett. 188, 205–208.
26 Pocotte, S.L. and Holz, R.W. (1986) J. Biol. Chem. 261, 1873–1877.
27 Morita, K., Brocklehurst, K.W., Tomares, S.M. and Pollard, H.B. (1985) Biochem. Biophys. Res. Commun. 129, 511–516.
28 Brocklehurst, K.W. and Pollard, H.B. (1986) Biochem. Biophys. Res. Commun. 140, 990–998.
29 Knight, D.E. and Baker, P.F. (1983) FEBS Lett. 160, 98–100.
30 Brocklehurst, K.W. and Pollard, H.B. (1985) FEBS Lett. 183, 107–110.
31 Brocklehurst, K.W. and Hutton, J.C. (1984) Biochem. J. 220, 283–290.
32 Hutton, J.C., Peshavaria, M. and Brocklehurst, K.W. (1984) Biochem. J. 224, 483–490.
33 Turgeon, J.L. and Cooper, R.H. (1986) Biochem. J. 237, 53–61.
34 Wrenn, R.W. (1984) Biochim. Biophys. Acta 775, 1–6.
35 Michener, M.L., Dawson, W.B. and Creutz, C.E. (1986) J. Biol. Chem. 261, 6548–6555.
36 Creutz, C.E., Dowling, L.G., Kyger, E.M. and Franson, R.C. (1985) J. Biol. Chem. 260, 7171–7173.