Activation of Annexin 7 by Protein Kinase C in Vitro and in Vivo*

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Annexin 7, a Ca\(^{2+}\)/GTP-activated membrane fusion protein, is preferentially phosphorylated in intact chromaffin cells, and the levels of annexin 7 phosphorylation increase quantitatively in proportion to the extent of catecholamine secretion. Consistently, various protein kinase C inhibitors proportionately reduce both secretion and phosphorylation of annexin 7 in these cells. In vitro, annexin 7 is quantitatively phosphorylated by protein kinase C to a mole ratio of 2.0, and phosphorylation is extraordinarily sensitive to variables such as pH, calcium, phospholipid, phorbol ester, and annexin 7 concentration. Phosphorylation of annexin 7 by protein kinase C significantly potentiates the ability of the protein to fuse phospholipid vesicles and lowers the half-maximal concentration of calcium needed for this fusion process. Furthermore, other protein kinases, including cAMP-dependent protein kinase, cGMP-dependent protein kinase, and protein-tyrosine kinase pp60c-src, also label annexin 7 with high efficiency but do not have this effect on membrane fusion. In the case of pp60c-src, we note that this kinase, if anything, modestly suppresses the membrane fusion activity of annexin 7. These results thus lead us to hypothesize that annexin 7 may be a positive mediator for protein kinase C action in the ectocytotic membrane fusion reaction in chromaffin cells.

Protein kinase C (PKC)† and possibly other protein kinase activators are believed to play a regulatory role in exocytotic secretion of hormones and neurotransmitters. Indeed, activation of PKC in bovine chromaffin cells, for example, with tumor-promoting phorbol esters (1, 2), or other secretagogues (3, 4), causes an increase in catecholamine secretion in a Ca\(^{2+}\)-dependent manner. By contrast, secretion is reduced when PKC activity is down-regulated by 24-h pretreatment with phorbol esters (5) or inhibited using various PKC inhibitors (6–8). The stimulatory effect of PKC activation on exocytosis has also been reported in various other cell types, including platelets (9), neutrophils (10), pituitary cells (11), insulin-secreting cells (12, 13), and mast cells (14, 15). Although phenomenologically well known, the specific sites of action of PKC in the stimulus-secretion cascade remain unknown.

The SNARE hypothesis has been proposed to explain the interactions between vesicle and plasma membranes during the period preceding exocytosis (16). In this model, a Ca\(^{2+}\)-independent core complex is formed between plasma membrane protein syntaxin and SNAP-25 and the synaptic vesicle protein synaptobrevin/VAMP. Vesicular synaptotagmin is identified as a low affinity Ca\(^{2+}\) sensor for subsequent exocytosis (17). Additional evidence suggests that trans-SNARE pairing may precede membrane fusion but is not be required during fusion (18–21). In addition, the preceding interaction of SNAP-25 with syntaxin is found to enhance the interaction between syntaxin and synaptobrevin/VAMP, suggesting that SNAP-25 regulates the formation of the SNARE complex (22). However, it has recently been reported that the phosphorylation of SNAP-25 by PKC actually decreases the interaction between syntaxin and SNAP-25. Thus, PKC makes the formation of the SNARE complex less likely. These data therefore suggest that the positive action of PKC on exocytosis is not likely to be mediated by SNARE proteins (23).

Alternatively, annexins have also been considered as possible mediators of exocytosis. Annexin 1 (ANX1), annexin 2 (ANX2), and annexin 7 (ANX7), which are members of the annexin family, have the ability to aggregate and fuse lipid vesicles (24). Such a result has been interpreted to suggest that they might play a role in regulating membrane fusion. Indeed, both ANX1 and ANX2 are found to be phosphorylated by PKC both in vivo (25, 26) and in vitro (27–29). However, phosphorylation of these proteins by PKC markedly inhibits their aggregation and fusion activities in vitro (27–29), indicating that they are also unlikely to mediate the positive action of PKC on exocytosis.

Annexin 7 (ANX7; synexin), which fuses membranes in a Ca\(^{2+}\)-dependent manner (30–33), has properties that have led us to give fuller credence to the possibility of its involvement in exocytosis. We have recently reported that ANX7 is a Ca\(^{2+}\)-activated GTPase, both in vitro and in vivo, and that its GTPase activity is increased in secreting chromaffin cells (34). More recently, we have reported that the heterozygous knock-out anx7 (+/−) mouse suffers from an insulin secretion deficit from islets of Langerhans, as well as defective Ca\(^{2+}\) signaling processes in β-cells (35). In addition, a homology analysis of anx7 has suggested to us the likelihood that this protein might be a target for PKC (36) and, therefore, a candidate for mediation of PKC action during exocytosis.

In this study, we report that ANX7 is phosphorylated in stimulated bovine chromaffin cells, and the level of ANX 7 phosphorylation is well correlated with the release of catecholamines. ANX7 is also phosphorylated in vitro by various...
kinesins, including PKC, cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein-tyrosine kinase pp60src. Significantly, only PKC-dependent phosphorylation of ANX7 enhances the membrane fusion activity of the protein, whereas phosphorylation by other kinesins does not affect this activity, or may even decrease it, as in the case of pp60src. Thus, the selective activation by PKC on exocytosis in vivo and the activation of ANX7 membrane fusion in vitro suggests that ANX7 may act as a positive mediator for PKC for the exocytotic membrane fusion reaction in chromaffin cells.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Chromaffin Cells—Chromaffin cells were isolated from bovine adrenal glands by collagenase digestion and purified on a Percoll gradient as described previously (37). Isolated cells were further purified by a selective plating method (38) and maintained in a CO₂ incubator under 5% CO₂/95% air.

(3P)Orthophosphoric Acid Labeling and Treatment of Chromaffin Cells with Secretagogues, PKC Activator, and Inhibitors—Cultured chromaffin cells (5 x 10⁹/dish, Falcon, 35 mm) were labeled with [³²P] orthophosphate (0.1 mCi/ml; Amersham Pharmacia Biotech) in phosphate-free Eagle’s minimum essential medium containing 10% dialyzed fetal bovine serum for 8 h at 37 °C (39). Then, the cells were stimulated with extracellular buffer A (buffer B without 2.2 mM CaCl₂ added). The cells were then stimulated for 30 min with 100 nM PMA, or 10 μM sodium deoxycholate, 5 mM EGTA, 0.2 mM Na₃VO₄, 1 mM MβCD, 100 nM MPA, 400 μg/ml PS liposomes, 0.05 unit (0.035 μg) of PKC, and 0.25 μg of purified human recombinant ANX7. All reactions were then incubated for 30 min. In other assays to determine the conditions of ANX7 phosphorylation, 0.25 μg of ANX7 was incubated for 60 min with or without 0.05 unit of PKC in 25 mM Pipes (pH 6.8), 10 mM MβCD, and the following conditions: no lipid or Ca²⁺ added; 1 mM Ca²⁺ added, without lipid; lipid added, without Ca²⁺; or both Ca²⁺ and lipid added. These conditions were also examined in the presence of 100 μM PKA. To determine the mode ratio of ANX7 phosphorylation by PKC, ANX7 (0.25 μg) was incubated with 0.05 μg of PKC under the optimal phosphorylation condition (25 mM Pipes (pH 6.8), 10 mM MβCD, 100 nM MPA, and 400 μg/ml PS liposomes). In the assay to determine the optimal ANX7 concentration for phosphorylation, ANX7 (0.05, 0.1, 0.25, 0.5, and 1 μg) was incubated for 60 min with 0.05 unit of PKC under the optimal phosphorylation condition. The reactions containing 1 μg ANX7 were further incubated for 90, 120, and 180 min. In the assay to determine ANX7 phosphorylation as a function of Ca²⁺ concentration, ANX7 (0.25 μg) was incubated for 30 min with 0.05 unit of PKC in 25 mM MES (pH 6.1) or 25 mM Pipes (pH 6.8), 10 mM MβCD, 100 nM MPA, 400 μg/ml PS liposomes, and the different free Ca²⁺ concentrations (0.01, 0.05, 0.1, 0.25, 0.5, and 1 mM). Free Ca²⁺ concentrations were determined as described (42). The above reactions were initiated by the addition of 2 mM of [γ-³²P]ATP in a final concentration of 0.1 mM (3000–4000 cpm/pmol; Amersham Pharmacia Biotech) and stopped by the addition of the SDS-PAGE sample buffer. The phosphorylation products were analyzed by SDS-PAGE and PhosphorImager analysis or autoradiography.

As for ANX7 phosphorylation by PKC, PKA, or pp60src, the assays were performed at 30 °C in a final volume of 30 μl as described (44–46). Purified human recombinant ANX7 (0.25 μg) was incubated for 30 min with 100 μM MβCD, 10 mM Pipes (pH 6.1), 10 mM MβCD, and 100 nM MPA. All phosphorylation reactions were initiated and analyzed as described above for the PKC reactions.

Phosphoamino Acid Analysis—The phosphoamino acid analysis was performed as described (47). After autoradiography, labeled ANX7 bands were excised from the SDS-PAGE gel and then electroeluted according to the manufacturer’s instructions (Bio-Rad). The eluate was dialyzed overnight in water to remove SDS. The dialyzed sample was concentrated by lyophilization and then reconstituted in 6 n HCl, followed by incubation at 110 °C for 2 h. O-Phosphoserine, O-phosphothreonine, and O-phosphotyrosine (Sigma Chemical Co.) at 5 μM each were added to the sample, and a 5-μl aliquot was spotted on a thin layer cellulose plate (Merck), followed by electrophoresis at 1 kV for 30 min in a pH 3.5 buffer (pyridine/acetate acid/water, 1:33:40, v/v). Unlabeled phosphoamino acids were stained with ninhydrin (0.2% in acetone), and labeled phosphoamino acids were detected using the PhosphorImager.
Lipid Vesicle Fusion Mediated by ANX7—The PS lipid vesicle fusion assay was performed as previously described (37). Lipid vesicles were first diluted to an A_{540} of 0.6 in fusion reaction buffer (0.3 M sucrose, 0.5 mM histidine (pH 6.1), 0.5 mM MgCl₂ and 0.1 mM EDTA). Phosphorylated or unphosphorylated ANX7 (0.5 μM) was incubated with 0.5 μl of lipid vesicle suspension in a final volume of 1 μl of fusion reaction buffer. Fusion was initiated by the addition of 1 μM Ca²⁺ (pH 6.1), ionomycin, and then measured by the change in the turbidity at absorbance of 540 nm (A_{540}) using a recording Hewlett-Packard spectrophotometer for 20 min at room temperature. For the Ca²⁺-dependent lipid vesicle fusion reaction mediated by phosphorylated or unphosphorylated ANX7, similar reactions were carried out as described above. Fusion was initiated by the addition of the indicated final Ca²⁺ concentrations (0.01, 0.05, 0.4, and 1 mM) and then monitored spectrophotometrically for 20 min. Free Ca²⁺ concentrations were determined as described elsewhere (43) and verified using a Ca²⁺-selective electrode.

Phosphorylation and Fusion Reaction—Simultaneous phosphorylation and lipid vesicle fusion reactions were carried out as described elsewhere (29). The reaction in a final volume of 1 ml contained 1 μg of ANX7, 0.5 unit of PKC, 0.3 M sucrose, 0.5 mM histidine (pH 6.1), 1 mM MgCl₂, 100 mM PMA, 100 μM ATP, and 0.5 mM lipid vesicle suspension. Controls were carried out in the absence of added ATP. Fusion and phosphorylation were simultaneously initiated by the addition of 1 mM Ca²⁺ at room temperature. Fusion was measured for 30 min as described above. To confirm that ANX7 phosphorylation occurred during fusion, parallel experiments were carried out in the presence of [γ-32P]ATP, and the stoichiometry was measured as described above.

Fusion and phosphorylation reactions in the presence of other kinases were carried out as described for PKC experiments above, except no PMA was added and PKC was replaced by 2000 units of PKG (plus 10 μM cGMp), 500 units of PKAcat, or 100 units of pp60 c-src.

Statistical Analysis—Data are presented as means ± S.D. A relationship between ANX7 phosphorylation and catecholamine secretion was assessed by a linear regression analysis (y axis, mean value of ANX7 phosphorylation induced by PMA, carbachol, or nicotine, and inhibited by various PKC inhibitors; x axis, mean value of catecholamine secretion under similar conditions as in phosphorylation). The statistical significant values (p) were determined by Student’s t test.

RESULTS

In Vivo Phosphorylation of ANX7 and Stimulation of Catecholamine Release from Chromaffin Cells—Using intact bovine adrenal chromaffin cells, we investigated whether ANX7 is phosphorylated under a variety of pro-secretory conditions, including treatment with PMA, carbachol, and nicotine. In these experiments, [32P] labeled cells were stimulated for 30 min with 100 nM PMA, 100 μM carbachol, 10 μM nicotine, or extracellular buffer B (control), and labeled endogenous ANX7 was immunoprecipitated with monoclonal antibody 10E7, followed by SDS-PAGE and PhosphoImager analysis. As shown in Fig. 1 (AI and BI), stimulation of cells with buffer B (control) results in a small amount of [32P] incorporation into ANX7. In contrast, labeling of ANX7 is markedly increased by about 3- to 5-fold for all agonists tested (Fig. 1, AI (bar 2) and BI (bars 2 and 5)).

As a further test for the involvement of PKC in the phosphorylation process, we examined whether the in vitro phosphorylation of ANX7 could be inhibited by various PKC inhibitors prior to stimulation with PMA or with other secretagogues. For these experiments we chose not only the relatively nonspecific staurosporine (48) but also the more selective calphostine C (49) and chelerythrine (50). As shown in Fig. 1A, all three inhibitors substantially reduce labeling of immunoprecipitated ANX7 from cells stimulated with 100 nM PMA. Staurosporine, at concentrations of 50, 100, and 200 nM, causes 35 ± 5, 54 ± 3, and 64 ± 7% inhibition of ANX7 labeling (mean ± S.D., n = 3), respectively (bars 3–5). In addition, calphostine C, at concentrations of 50 and 500 nM, causes 45 ± 7 and 64 ± 3% inhibition of ANX7 labeling, respectively (bars 6 and 7), whereas chelerythrine, at concentrations of 0.7 and 1 μM, also causes 42 ± 11 and 58 ± 16% inhibition of ANX7 labeling, respectively (bars 8 and 9). Furthermore, calphostine C and chelerythrine both also cause a substantial reduction in labeling of immunoprecipitated ANX7 from cells stimulated with carbachol or nicotine (Fig. 1BI). In these experiments, 50 nM calphostine C and 0.7 μM chelerythrine cause 44 ± 2 and 45 ± 7% inhibition in carbachol-induced labeling of ANX7, respectively (bars 3 and 4), and these two inhibitors at the same concentrations also cause 46 ± 6 and 49 ± 4% inhibition in nicotine-induced labeling of ANX7, respectively (bars 6 and 7).

We further examined whether phosphorylation of ANX7 in vivo could be correlated with catecholamine secretion under the above conditions. Unlabeled cells were preincubated for 1 h in the presence or absence of 100 nM staurosporine, 50 nM calphostine C, or 0.7 μM chelerythrine. The cells were then stimulated with or without 100 nM PMA, 100 μM, or 10 μM nicotine for 30 min. After incubation, the medium from each well was collected and assayed for secreted catecholamines. As shown in Fig. 1AI and 1BI, incubation with PMA, carbachol, or nicotine results in a 3.5-, 5.3-, or 6.0-fold increase in catecholamine secretion, respectively (Fig. 1, AI and BI (bar 2) and BI (bars 2 and 5)). By contrast, preincubation with staurosporine, calphostine C, or chelerythrine only results in a 1.5-, 1.6-, or 1.4-fold increase in PMA-induced secretion, respectively (Fig. 1AII, bars 3–5). Likewise, calphostine C and chelerythrine also allow 2.7- and 2.9-fold increases in carbachol-induced secretion, respectively (Fig. 1BII, bars 3 and 4), and 3.1- and 3.2-fold increases in nicotine-induced secretion, respectively (Fig. 1BII, bars 6 and 7). Moreover, as shown in Fig. 1C, there appears to be a good correlation between the two processes, secretion and ANX7 phosphorylation (R² = 0.9622).

In Vitro Phosphorylation of ANX7—To determine whether ANX7 might be a substrate for PKC in vitro, we used purified rat brain PKC to phosphorylate purified recombinant ANX7 and analyzed the products by SDS-PAGE and PhosphoImager. As shown in Fig. 2, PKC indeed phosphorylates ANX7 in a highly efficient manner and is affected by a variety of extensive variables. ANX7 phosphorylation by PKC is somewhat dependent on pH between pH 6.1 and 7.5 (Fig. 2A). After 30 min of incubation at 30 °C, an apparent maximal level of ANX7 phosphorylation by PKC at pH 6.8 is achieved with a stoichiometry of 1.61 mol of P_i/mol of ANX7, respectively (Fig. 2A, inset bars 2–5). No phosphorylation of ANX7 is detected when ANX7 to PKC not on the activity of the PKC, per se.

At pH 6.8, phosphorylation of ANX7 is dependent on the presence of PKC, Ca²⁺, phospholipid, and the PKC activator PMA (Fig. 2B). No phosphorylation of ANX7 is detected when PKC is omitted from the reaction mixture (bar). Similar negative results are found when both Ca²⁺ and phospholipid are omitted from the reaction mixture containing PKC (bar). Furthermore, the presence of 1 mM Ca²⁺ alone, or phospholipid alone, is unable to support an optimal level of phosphorylation (bars 2 and 3). However, when both are present, the level of phosphorylation is greatly enhanced with a stoichiometry of 1.52 ± 0.02 mol of P_i/mol of ANX7 for 60 min (bar). Moreover, 100 nM PMA significantly enhances the level of PKC-catalyzed phosphorylation of ANX7 under the various conditions (compared bars 5–8 with bars 1–4, respectively).
Because we could vary the mole fraction of phosphorylation between 1 and 2, we then examined the kinetics of the process in greater detail (Fig. 2C). Under optimal experimental conditions, phosphorylation of ANX7 is complete after 60 min with a stoichiometry of 2.01 ± 0.01 mol of P /mol of ANX7 (mean ± S.D., n = 5). The rate of the phosphorylation reaction is also dependent on the ANX7 concentration. As shown in Fig. 2D, the efficiency of phosphorylation is decreased as the ANX7 concentration increases. At a higher ANX7 concentration (e.g. 1 μg/30 μl), however, the optimal level of phosphorylation is achieved only if the incubation time is extended (inset).

The fact that Ca2+ is absolutely required for ANX7 phosphorylation (see Fig. 2B) suggests either that Ca2+ is needed to activate only the Ca2+/phospholipid-dependent PKC activity, or that Ca2+ binding to ANX7 is a prerequisite for PKC-dependent phosphorylation. To distinguish between these two possibilities, we examined the Ca2+ dependence of ANX7 phosphorylation by PKC at both pH 6.1 and 6.8 (Fig. 2E). As shown in the inset of Fig. 2E, the autophosphorylation level of PKC is essentially the same throughout the range of final Ca2+ concentrations tested (0.01–1.0 mM), at both pH conditions. By contrast, the mole ratio of ANX7 phosphorylation is increased as the Ca2+ concentration increases. At pH 6.8, the Ca2+ titration curve for PKC-dependent ANX7 phosphorylation is biphasic. A minimal saturated phosphorylation level with a stoichiometry of 0.5 mol of P /mol of ANX7 is observed throughout the lower range of Ca2+ concentrations (0.01–0.20 mM), and this level is eventually increased as the free Ca2+ concentration increases from 0.20 to 1.0 mM. On the other hand, the curve obtained at pH 6.1 is more sigmoidal. Under this pH condition, no phosphorylation of ANX7 is observed at any Ca2+ concentration below 0.05 mM, and ANX7 phosphorylation eventually increases as the Ca2+ concentration increases beyond 0.05 mM. Thus, the action of Ca2+ on ANX7 labeling efficiency appears to be on ANX7 rather than PKC.

Phosphoamino Acid Analysis—To further analyze the PKC reaction both in vivo and in vitro, we performed phosphoamino acid analysis of ANX7 immunoprecipitated from 32P-labeled chromaffin cells. We also examined ANX7 phosphorylated in vitro by purified rat brain PKC. After carbachol or nicotine
In *vitro* phosphorylation of ANX7 by protein kinase C. A, pH dependence. ANX7 (0.25 μg) was incubated at 30 °C with 0.05 unit of PKC in 25 mM Tris-HCl (pH 7.5), 25 mM PIPES (pH 6.8), or 25 mM MES (pH 6.1), 10 mM MgCl₂, 1 mM CaCl₂, 100 mM PMA, 400 μg/ml PS liposomes, and 100 μM [γ-32P]ATP. *, p < 0.05, compared with pH 6.1; †, p < 0.005, compared with pH 7.5. B, ANX7 (0.25 μg) was incubated with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM MgCl₂, 100 mM [γ-32P]ATP, and various conditions as described in the text. C, ANX7 (0.25 μg) was incubated with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM MgCl₂, 1 mM CaCl₂, 100 mM PMA, and 400 μg/ml PS liposomes. The reactions were stopped at the indicated time intervals after the addition of 100 μM [γ-32P]ATP. D, various indicated ANX7 concentrations were incubated for 60 min with 0.05 unit of PKC under the optimal phosphorylation condition (pH 6.8) in a final volume of 30 μl. The inset shows the extended time course of phosphorylation of ANX7 at 1.0 μg. E, ANX7 (0.25 μg) and PKC (0.05 unit) were incubated for 30 min in the pH 6.1 (filled circles) or pH 6.8 (empty circles) phosphorylation buffer containing the indicated free Ca²⁺ concentrations. In A, B, C, and E, the inset shows a representative PhosphorImager data of three to five different experiments. All data are the mean ± S.D. (n = 3–5) and are expressed in mole ratios.

In Fig. 4B, as the reaction progresses, the relative rate of the fusion reaction containing ATP is greatly enhanced, as compared with that of the control (minus ATP). In parallel control experiments, in which ANX7 has been omitted from the reaction mixture containing either ATP or no ATP, the addition of PKC is unable to induce fusion of lipid vesicles (data not shown). Similarly, the addition of PMA is also unable to alter the fusion reaction induced by ANX7 (data not shown). These two results (Fig. 4, A and B) are thus consistent with each other and suggest that access to phosphorylated ANX7 is a rate-limiting step for efficient activation of membrane fusion.

**Ca²⁺ Dependence of Fusion Reactions Induced by Phosphorylated and Unphosphorylated ANX7**—Because the fusion of lipid vesicles by ANX7 is dependent on Ca²⁺, we then examined the effect of phosphorylation by PKC on the Ca²⁺ dependence of this fusion process. In these experiments, phosphorylated and unphosphorylated ANX7 were prepared as described above (see Fig. 4A), and their fusion activities were examined at different final Ca²⁺ concentrations, ranging from 0.01 to 1.0 mM. As shown in Fig. 5A, the rate of lipid vesicle fusion induced by phosphorylated ANX7 is markedly increased at lower Ca²⁺ concentrations, as compared with the same process induced by unphosphorylated ANX7 (p < 0.005). In addition, not only is

**Lipid Vesicle Fusion by Phosphorylated ANX7**—To study the effect of PKC-dependent phosphorylation on a relevant *in vitro* activity of ANX7, we chose to examine the lipid vesicle fusion reaction mediated by this protein. Two parallel experimental strategies were employed. In one experiment, ANX7 was prephosphorylated with PKC in the presence or absence of ATP, followed by extraction from the reaction mixture, and these phosphorylated and unphosphorylated forms were used to initiate the membrane fusion reaction (Fig. 4A). Fig. 4A shows a time course of the fusion of lipid vesicles catalyzed by either phosphorylated or unphosphorylated ANX7. When the fusion reaction is initiated with 1 mM Ca²⁺, the rate and the extent of lipid vesicle fusion induced by phosphorylated ANX7 is markedly enhanced over that of the unphosphorylated ANX7.

In the second experiment, PKC was added simultaneously with ANX7 in the presence or absence of added ATP. As shown
the potency of the reaction increased by PKC phosphorylation of ANX7, but the efficacy is also increased. Thus, there is a significant difference between the fusion processes mediated by phosphorylated ANX7 and its unphosphorylated form with the specific consequence of phosphorylation increasing the efficacy and extent of Ca\(^{2+}\) activation.

As shown in Fig. 5B, the Ca\(^{2+}\) concentration required to induce half-maximal fusion activity (50\% of \(F_{\max}\)) is 200 \(\mu M\) for the unphosphorylated protein, which is in accord with previous reports (34, 51). By contrast, when ANX7 is phosphorylated by PKC, at the same protein concentration, this value is lowered to \(-50 \mu M\). Thus, Ca\(^{2+}\) not only potentiates the susceptibility of ANX7 to labeling by PKC (see Fig. 2E), but PKC action also raises the affinity of ANX7 for Ca\(^{2+}\).

It was also possible that the enhancement of fusion by PKC might be due to an increase in lipid binding activity. To test this hypothesis we examined the lipid binding properties of phosphorylated and unphosphorylated ANX7. Following a 20-min fusion reaction, the mixture was centrifuged at 100,000 \(\times\) g, and the protein bound to lipid vesicles was quantified by SDS-PAGE. The inset of Fig. 5A shows the recovery of both phosphorylated and unphosphorylated forms of ANX7 from the lipid pellets incubated at different Ca\(^{2+}\) concentrations. As shown by the figure, phosphorylation did not increase the amount of protein recovered with lipid vesicles. Thus, binding of ANX7 to membranes depends exclusively on Ca\(^{2+}\); however, the efficiency by which the Ca\(^{2+}\)-induced membrane binding step of ANX7 is converted into membrane fusion depends on PKC.

In Vitro Phosphorylation of ANX7 by Other Kinases and Their Effects on ANX7-driven Lipid Vesicle Fusion—As a control for the specificity of PKC on ANX7 activity, we also examined the phosphorylation of ANX7 by other kinases, including purified PKG, PKA, and pp60\(^{src}\), each with an optimal enzymatic activity (Fig. 6A). Based on the calculation of ANX7 phosphorylation by these kinases, the amounts of phosphate...
incorporated into ANX7 were 0.7, 1.0, and 0.9 mol/mol of ANX7 when incubated for 60 min with PKG, PKA, and pp60src, respectively (data not shown).

Based on these conditions, we then tested the consequences of phosphorylation by PKA, PKG, or pp60src for ANX7 activity on membrane fusion. Using the methods developed to study the PKC effect (see Fig. 4B), ANX7 was incubated in the presence of lipid vesicles and PKA, PKG plus cGMP, or pp60src, with or without added ATP. As shown in Fig. 6B, PKA or PKG phosphorylation has no effect on the membrane fusion activity of ANX7. In contrast to the PKC-mediated effect, we observed a modest decrease in the rate of fusion activity in the reaction containing pp60src and ATP.

**DISCUSSION**

In this study, we present, for the first time, evidence that stimulation of intact bovine chromaffin cells with phorbol ester PMA, carbachol, or nicotine markedly increases the phosphorylation of endogenous ANX7 (Fig. 1). Furthermore, using PKC inhibitors with both selective and relatively nonselective properties, we also show that the levels of PKC-dependent labeling of endogenous ANX7 are closely correlated with the levels of catecholamine secretion. These results indicate that ANX7 phosphorylation in vivo appears to be mediated by this kinase. Equivalent studies in vitro show that ANX7 is a quantitative substrate for PKC (Fig. 2) and that PKC phosphorylation enhances the Ca2+-dependent membrane fusion driven by ANX7 (Figs. 4 and 5). These findings strongly imply that ANX7 is one of the potential phosphoproteins involved in the exocytotic machinery in chromaffin cells and possibly in other cell types. These conclusions are further supported by our recent report that a nullizygous (−/−) knockout of the anx7 gene in mouse is lethal and that insulin secretion from islets of Langerhans of the heterozygous knockout anx7 (+/−) mouse is defective (35).

**Ca2+ and pH Action on ANX7 Control the Efficiency of Phosphorylation by PKC**—The limiting factor for the ANX7 phosphorylation event appears to be the structural conformation of the ANX7 protein itself. The efficiency of in vitro phosphorylation of ANX7 by PKC is somewhat dependent on pH with an optimal pH of pH 6.8 (Fig. 2A). This effect of pH on ANX7 phosphorylation is not attributed to the pH-dependent activity of PKC itself, because the optimal pH of PKC activation is known to be at pH 7.5 (52). Rather, it is likely that ANX7 phosphorylation site(s) become more accessible to PKC at this pH range. Circular dichroism studies of recombinant ANX7 have indicated substantial conformational flexibility over the pH interval of 6.5–7.5. The *in vitro* pH condition (pH 6.8) used to yield an optimal ANX7 phosphorylation appears to be in accord with the cytosolic pH of the chromaffin cell. For instance, several previous studies have shown that the Ca2+-dependent catecholamine secretion is increased at low pH with an optimal pH around pH 6.6 (53) and that the cytosolic pH of the chromaffin cell is transiently acidified upon stimulation by acetylcholine or nicotine (54).

Our data also support the concept that Ca2+ modifies the conformation of ANX7 to permit enhanced labeling by PKC (see Fig. 2E). The evidence is that, although autophosphorylation of PKC remains unchanged, ANX7 phosphorylation is increased significantly as the free Ca2+ concentration elevates from 10 μM to 1 mM. The pH of the medium also dictates the pattern of Ca2+-dependent phosphorylation of ANX7, either having a biphasic dose-response curve (at pH 6.8) or a sigmoidal curve (at pH 6.1). Thus, the elevated Ca2+ concentration and the slightly acidic pH, both of which are observed to change coincidentally in the cell during stimulation, appear synergistically to induce the structural conformations of ANX7 that enhance the *in vitro* phosphorylation reaction.

**PKC Activates ANX7-driven Membrane Fusion in Vitro**—The ANX7-driven membrane fusion reaction is a well-established *in vitro* model for exocytosis (30–33). The results shown in Figs. 4 and 5 suggest that the lipid binding and fusion activities of ANX7 are separable functions and that only the fusion activity of the protein is regulated by PKC. Phosphorylation of ANX7 by PKC markedly increases the lipid vesicle fusion activity, and significantly lowers the half-maximal Ca2+ concentration needed for ANX7-induced lipid vesicle fusion. PKC confers a K1/2(app) of 50 μM for phosphorylated ANX7 as opposed to 200 μM for the unphosphorylated form. However, both phosphorylated and unphosphorylated ANX7 are found to bind to lipid vesicles with equivalent affinities as a function of free Ca2+ concentration (*inset* of Fig. 5). Only the phosphorylated protein, however, is able to induce lipid vesicle fusion at lower Ca2+ concentrations (≤50 μM). At present, the mechanism by which the fusion activity of ANX7 is enhanced by PKC remains to be

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**Fig. 5. Ca2+ dependence of fusion of lipid vesicles by phosphorylated and unphosphorylated ANX7.** A, phosphorylated (empty circles) or unphosphorylated (filled circles) ANX7 (0.5 μg), prepared as described in Fig. 4A, was added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM MgCl2, 0.1 mM EGTA, and 0.5 ml of lipid vesicle suspension. Fusion was initiated by the addition of the indicated final Ca2+ concentrations and measured by the change in absorbance at 540 nm after 20 min. Data are the mean ± S.D. (n = 3). *, p < 0.005, compared with the control (nANX7). To determine lipid binding by ANX7, the lipid vesicles were centrifuged after the fusion reaction was complete, and the lipid-associated protein was analyzed by SDS-PAGE. The inset shows phosphorylated ANX7 (pANX7, ○) and its unphosphorylated form (nANX7, ●) that cosedimented with the lipid vesicles at indicated free Ca2+ concentrations. B, data from A were replotted to highlight the increasing affinity of ANX7 for Ca2+ by PKC phosphorylation. The Vmax of each curve was determined by a Lineweaver-Burk plot and then used as 100% maximal fusion activity. Based on these Vmax values, the original data were transformed, expressed as a percentage of maximum of fusion activity, and replotted as shown.
fully elucidated. Several studies have suggested that annexin self-association, after binding to the membrane, may be required to allow the annexins to aggregate and fuse lipid vesicles (55, 56). Therefore, it is reasonable to anticipate that phosphorylation of ANX7 by PKC may potentiate the intermolecular interactions occurring between ANX7 molecules, resulting in the enhancement of membrane fusion. ANX7 can therefore be usefully hypothesized as part of the Ca\textsuperscript{2+} signal transduction pathway.

In conclusion, we have demonstrated that ANX7 serves as the substrate for PKC and certain other kinases. Only PKC-dependent phosphorylation has a positive effect on the in vitro membrane fusion model of exocytosis. The specific action involving lowering the K\textsubscript{app} for Ca\textsuperscript{2+} from 200 μM to 50 μM. Consistently, stimulation of chromaffin cells with PKC activators indeed results in phosphorylation of endogenous ANX7 concomitantly with the release of catecholamines. These results thus support the hypothesis that ANX7 is a site of action for PKC activation during exocytosis.

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**FIG. 6. In vitro phosphorylation of ANX7 by various kinases and their effects on the fusion activity of ANX7.**

A. ANX7 (0.25 μg) was incubated for 30 min at 30 °C with 200 units of PKG plus 10 μM cGMP, 50 units of catalytic subunit of PKA, or 10 units of pp60src in 25 mM MES (pH 6.1), 10 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, and 100 μM [γ-\textsuperscript{33P}]ATP. The result shown is a representative PhosphorImager data of four different experiments. Arrows indicate the position of ANX7. Other labeled bands shown are PKG (*), PKA (†), and pp60\textsuperscript{src} (‡). B. ANX7 (1 μg) and 2000 units of PKG plus 10 μM cGMP (empty triangles), 500 units PKA (empty squares), or 100 units pp60\textsuperscript{src} (empty circles) were added to a 1-mL reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM MgCl\textsubscript{2}, 100 μM ATP, and 0.5 ml of lipid vesicle suspension. Controls were carried out in the absence of added ATP (filled circles). Phosphorylation and fusion reactions were initiated simultaneously by the addition of 1 mM Ca\textsuperscript{2+} at room temperature. Fusion was measured by the change in absorbance at 540 nm after 30 min. Data are the mean ± S.D. (n = 3).
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J. Biol. Chem. 2001, 276:12813-12821.
doi: 10.1074/jbc.M008482200 originally published online January 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M008482200

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