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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 $pp60^{c\text{-}src}$, also label annexin 7 with high efficiency but do not have this effect on membrane fusion. In the case of $pp60^{c\text{-}src}$, 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 exocytotic membrane fusion reaction in chromaffin cells.

Protein kinase C (PKC)[1] 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 knockout 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

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[1] The abbreviations used are: PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; P_i, inorganic phosphate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; PAGE, polyacrylamide gel electrophoresis; SNAP-25, 25-kDa synaptosome-associated protein; VAMP, vesicle-associated membrane protein; SNARE, soluble NSF-attachment protein receptor.

kinases, including PKC, cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein-tyrosine kinase pp60$^{c\text{-}src}$. Significantly, only PKC-dependent phosphorylation of ANX7 enhances the membrane fusion activity of the protein, whereas phosphorylation by other kinases does not affect this activity, or may even decrease it, as in the case of pp60$^{c\text{-}src}$. 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 of 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 CO_2 incubator under 5% CO_2/95% air.

[^{33}P]Orthophosphoric Acid Labeling and Treatment of Chromaffin Cells with Secretagogues, PKC Activator, and Inhibitors—Cultured chromaffin cells (5×10^6/dish, Falcon, 35 mm) were labeled with [^{33}P]P_i (0.1 mCi/ml; Amersham Pharmacia Biotech) in phosphate-free Eagle's minimal essential medium containing 10% dialyzed fetal calf serum for 8 h at 37 °C (39). Then, the cells were washed once with Ca^{2+}-free extracellular buffer A (buffer B without 2.2 mM $CaCl_2$ added). The cells were stimulated with extracellular buffer B (118 mM NaCl, 4.2 mM KCl, 10 mM $NaHCO_3$, 10 mM glucose, 25 mM Hepes (pH 7.2), 0.1% bovine serum albumin, 1.2 mM $MgCl_2$, and 2.2 mM $CaCl_2$) containing 100 nM phorbol 12-myristate 13-acetate (PMA; ICN), 100 μM carbachol (Sigma) or 10 μM nicotine (Sigma) for 30 min at 37 °C. After incubation, the cells were rapidly washed twice with buffer A and then solubilized in lysis buffer for immunoprecipitation. For experiments with PKC inhibitors, ^{33}P-labeled cells were preincubated for 60 min at 37 °C with or without staurosporine (50, 100, and 200 nM, Calbiochem), calphostine C (50 and 500 nM; Calbiochem), or chelerythrine (0.7 and 1.0 μM, Calbiochem) in buffer A. The cells were then stimulated for 30 min with 100 nM PMA, 100 μM carbachol, or 10 μM nicotine, followed by cell lysis for immunoprecipitation. Control experiments were performed using cells incubated with buffer B or buffer B containing Me_2SO (for experiments using Me_2SO-soluble compounds, *e.g.* PMA, staurosporine, calphostine C, and chelerythrine). For determining catecholamine secretion under the above conditions, parallel experiments were carried out using unlabeled chromaffin cells, and the media were then collected for measuring catecholamine concentrations.

Immunoprecipitation of ^{33}P-Labeled ANX7—The cells were lysed in 1 ml of ice-cold lysis buffer (150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 5 mM EGTA, 0.2 mM Na_3VO_4, 1 mM β-glycerolphosphate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl (pH 7.5)), followed by incubation for 20 min on ice. The lysates were clarified by centrifugation at 12,000 $\times$ *g* for 15 min at 4 °C, and the resulting lysates were precleared by incubation for 30 min with 50 μl of a 10% (v/v) suspension of protein G-Sepharose (Zymed Laboratories Inc.), followed by centrifugation. The final lysates, with equal protein amounts determined by a BCA kit (Pierce), were then incubated with 10 μg of anti-ANX7 monoclonal antibody 10E7 for 6 h at 4 °C. Immunoprecipitates were collected on protein G-Sepharose, washed four times by pelleting in cold lysis buffer, separated by SDS-PAGE, and analyzed by phosphorimaging (PhosphorImager, Molecular Dynamics) or autoradiography.

Measurement of Catecholamine Release—The assay for catecholamine release from chromaffin cells was performed exactly as described previously (37). The release of catecholamines was expressed as a percentage of total cellular catecholamines.

Preparation of Phosphatidylserine Lipid Vesicles—PS lipid vesicles were prepared fresh daily by the swelling method (40). Briefly, highly purified (>99%) brain phosphatidylserine (Avanti Polar Lipids) in a 1:4 chloroform-methanol solution was dried slowly under nitrogen and then allowed to swell in 0.3 M sucrose at room temperature. The suspension was then sonicated and centrifuged at 12,000 $\times$ *g*. The PS lipid vesicle pellet was resuspended in 0.3 M sucrose solution.

Isolation and Purification of Human Recombinant ANX7—Human recombinant ANX7 was isolated and purified as described previously (41). Briefly, *Escherichia coli* bacteria containing the anx7-expressing vector (pTrc-FLS) were grown in 1 liter of Luria broth at 37 °C. After reaching an A_{540} level of 0.6, the culture was incubated overnight in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (ICN). After incubation, the bacteria were harvested by centrifugation. Expressed recombinant ANX7 was then extracted from the *E. coli* paste, concentrated by precipitation with 0–20% (w/v) $(NH_4)_2SO_4$ and purified by gel filtration using Ultragel AcA54 (Biosphere). Partial purified ANX7 was further purified by binding to PS lipid vesicles in the presence of Ca^{2+} and extracting with EGTA. This purification step was repeated six times to finally yield a highly purified (≥98%) ANX7 preparation, determined by SDS-PAGE and silver staining.

In Vitro Phosphorylation of ANX7—All phosphorylation assays using purified rat brain PKC were performed at 30 °C in a final volume of 30 μl as described elsewhere (42). Rat brain PKC with a purity of ≥95% was purchased from Calbiochem and contained the PKC isoforms α, β, and γ. Phosphorylation of ANX7 by PKC was examined at different pH values, and the reaction mixture contained the following: 25 mM Tris-HCl (pH 7.5), 25 mM PIPES (pH 6.8) or 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 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 $MgCl_2$, and the following conditions: no lipid or Ca^{2+} added; 1 mM Ca^{2+} added, without lipid; lipid added, without Ca^{2+}; or both Ca^{2+} and lipid added. These conditions were also examined in the presence of 100 nM PMA. To determine the mole ratio of ANX7 phosphorylation by PKC, ANX7 (0.25 μg) was incubated for the indicated time periods with 0.05 unit of PKC under the optimal phosphorylation condition (25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 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 160 min. In the assay to determine ANX7 phosphorylation as a function of Ca^{2+} 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 $MgCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and the different free Ca^{2+} concentrations (0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, and 1 mM). Free Ca^{2+} concentrations were determined as described (43). The above reactions were initiated by the addition of 2 μCi of [γ-^{33}P]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 PKA, PKG, and pp60$^{c\text{-}src}$, the assays were carried out 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 10 units of pp60$^{c\text{-}src}$ (Calbiochem (44)), 50 units of catalytic subunit of PKA (Promega (45)) or 200 units of PKG (Promega (46)) plus 10 μM cGMP in 25 mM MES (pH 6.1), 10 mM $MgCl_2$, and 1 mM $CaCl_2$. 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 dialysate was concentrated by lyophilization and was then resuspended 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 mM 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/acetic 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.

Extraction of Phosphorylated and Unphosphorylated ANX7—Extraction of the phosphorylated and unphosphorylated protein from lipid vesicles was performed as described (29). In each of 30 reactions, 1 μg of ANX7 was incubated for 2 h at 30 °C with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and 0.1 mM ATP in a final volume of 30 μl. Unphosphorylated ANX7 was treated in a similar manner as described for phosphorylated ANX7, except ATP was omitted. All reactions were pooled and centrifuged at 100,000 $\times$ *g* for 10 min, and the pellet, containing lipid and lipid-associated ANX7, was resuspended in 25 mM Tris-HCl (pH 7.5), 20 mM EGTA, and 20 mM EDTA. The mixture was sonicated in a bath type sonicator and then incubated for 30 min on ice, followed by centrifugation at 100,000 $\times$ *g*. The supernatant, containing ANX7, was collected and recentrifuged twice to remove residual lipid vesicles. After

removing EGTA and EDTA, the concentration of phosphorylated and unphosphorylated ANX7 was determined by immunoblotting using ^{125}I-labeled secondary antibody and a known amount of ANX7 as a standard, and quantitated with the PhosphorImager. A parallel experiment using [γ-^{33}P]ATP was also carried out to determine the stoichiometry of phosphorylation.

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, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, and 0.1 mM EGTA). Phosphorylated or unphosphorylated ANX7 (0.5 μg) was incubated with 0.5 ml of lipid vesicle suspension in a final volume of 1 ml of fusion reaction buffer. Fusion was initiated by the addition of 1 mM $[Ca^{2+}]_{final}$ 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 Ca^{2+}-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 Ca^{2+} concentrations (0.01, 0.05, 0.4, and 1 mM) and then monitored spectrophotometrically for 20 min. Free Ca^{2+} concentrations were determined as described elsewhere (43) and verified using a Ca^{2+}-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, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 nM PMA, 100 μM ATP, and 0.5 ml of 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 $[Ca^{2+}]_{final}$ 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-^{33}P]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 PKA_{cat}, or 100 units of $pp60^{c\text{-}src}$.

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 using 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, ^{33}P-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 PhosphorImager analysis. As shown in Fig. 1 (*AI* and *BI*), stimulation of cells with buffer B (control) results in a small amount of ^{33}P 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 vivo* 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 nonselective staurosporine (48) but also the more selective calphostine C (49) and chelerythrine (50). As shown in Fig. 1*AI*, 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 48 ± 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. 1*BI*). 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. 1*AII* and 1*BII*, incubation with PMA, carbachol, or nicotine results in a 3.5-, 5.3-, or 6.0-fold increase in catecholamine secretion, respectively (Fig. 1, *AII* (*bar 2*) and *BII* (*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. 1*AII*, *bars 3–5*). Likewise, calphostine C and chelerythrine also allow 2.7- and 2.9-fold increases in carbachol-induced secretion, respectively (Fig. 1*BII*, *bars 3* and *4*), and 3.1- and 3.2-fold increases in nicotine-induced secretion, respectively (Fig. 1*BII*, *bars 6* and *7*). Moreover, as shown in Fig. 1*C*, there appears to be a good correlation between the two processes, secretion and ANX7 phosphorylation (R^2 = 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 human recombinant ANX7 and analyzed the products by SDS-PAGE and PhosphorImager. 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. 2*A*). 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 ± 0.13 mol of P_i/mol of ANX7 (mean ± S.D., n = 5). As compared with the pH 6.8 condition, the stoichiometries of ANX7 phosphorylation at pH 6.1 and 7.5 are 1.35 ± 0.17 and 1.17 ± 0.16 mol of P_i/mol of ANX7, respectively. By contrast, the level of autophosphorylation of PKC is relatively unchanged under these pH conditions (*inset* of Fig. 2*A*). Thus, the effect of pH appears to be on the susceptibility of 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, Ca^{2+}, phospholipid, and the PKC activator PMA (Fig. 2*B*). No phosphorylation of ANX7 is detected when PKC is omitted from the reaction mixture (*bar 9*). Similar negative results are found when both Ca^{2+} and phospholipid are omitted from the reaction mixture containing PKC (*bar 1*). Furthermore, the presence of 1 mM Ca^{2+} 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 4*). 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).

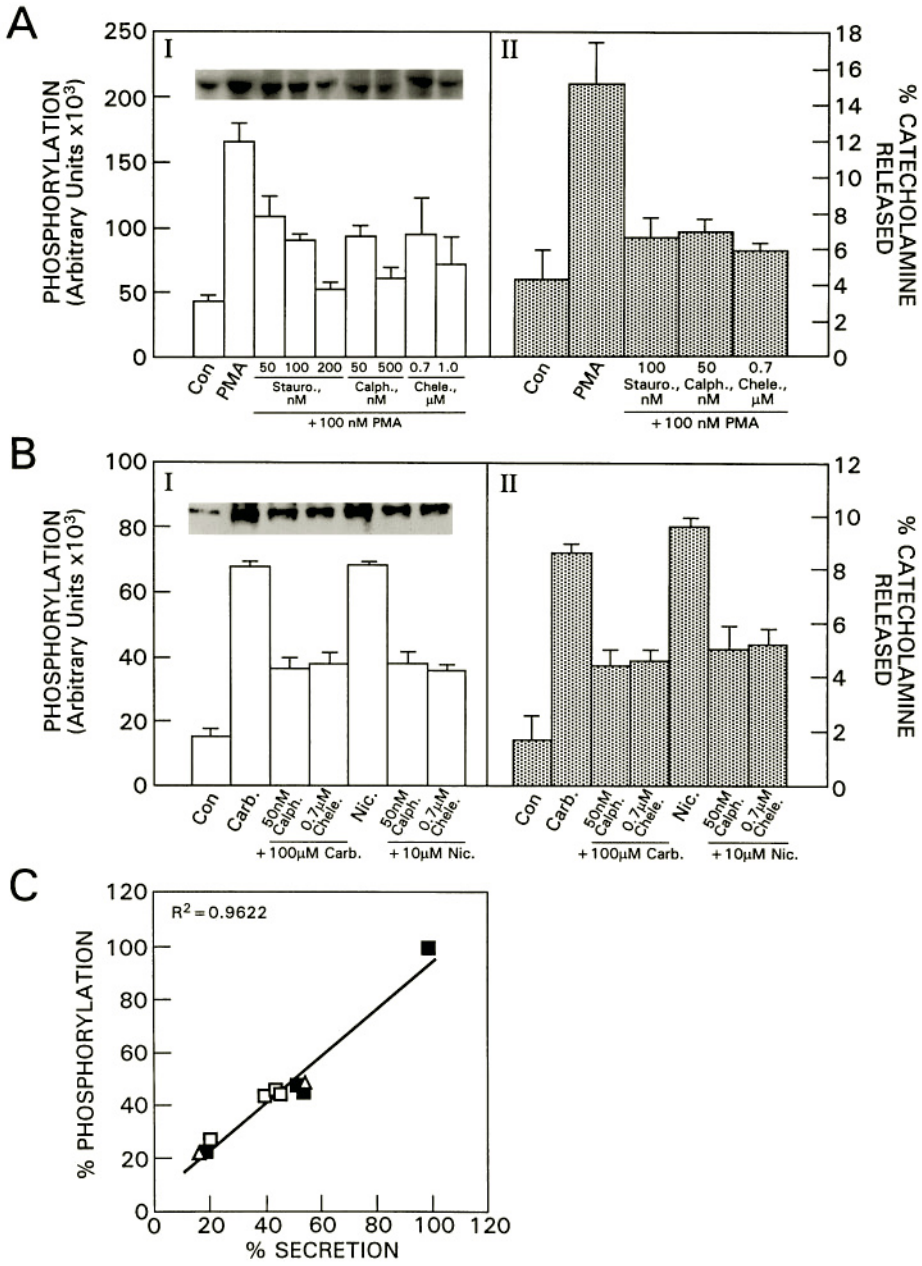

FIG. 1. **Effects of secretagogues, PKC activator and inhibitors on ANX7 phosphorylation and secretion of catecholamines from chromaffin cells.** *AI* and *BI*, ^{33}P-labeled cells were stimulated for 30 min at 37 °C with 100 nM PMA, 100 μM carbachol, 10 μM nicotine, or buffer B (control). In experiments using PKC inhibitors, labeled cells were preincubated for 60 min with or without staurosporine, calphostine C, or chelerythrine at indicated concentrations, followed by stimulation with PMA, carbachol, or nicotine. The cells were lysed in lysis buffer. The lysates, with equal amounts of total protein, were then incubated with anti-ANX7 antibody for 6 h at 4 °C. By Western blot analysis, equal amounts of the protein were found to be present on the blots under the various experimental conditions. Immunoprecipitation of ANX7 was analyzed by SDS-PAGE and PhosphorImager. The level of ^{33}P incorporation into ANX7 is reported as the arbitrary unit (mean ± S.D., n = 3–4; *open bars*). The *inset* shows a representative PhosphorImager data. *AII* and *BII*, to determine catecholamine secretion under the above conditions, parallel experiments were carried out using unlabeled chromaffin cells, and the media were collected for measuring catecholamine concentrations. The release is expressed as % of the total catecholamine content (mean ± S.D., n = 4; *speckled bars*). *C*, correlation between ANX7 phosphorylation and catecholamine secretion from chromaffin cells in response to various PKC inhibitors and PMA (*empty squares*), carbachol (*filled squares*), or nicotine (*empty triangles*) is shown. Correlation coefficient (R^2) and the *computer-fitted line* for all data points were obtained from the results described in *A* and *B*.

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. 2*C*). Under optimal experimental conditions, phosphorylation of ANX7 is complete after 60 min with a stoichiometry of 2.01 ± 0.01 mol of P_i/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. 2*D*, 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 Ca^{2+} is absolutely required for ANX7 phosphorylation (see Fig. 2*B*) suggests either that Ca^{2+} is needed to activate only the Ca^{2+}/phospholipid-dependent PKC activity, or that Ca^{2+} binding to ANX7 is a prerequisite for PKC-dependent phosphorylation. To distinguish between these two possibilities, we examined the Ca^{2+} dependence of ANX7 phosphorylation by PKC at both pH 6.1 and 6.8 (Fig. 2*E*). As shown in the *inset* of Fig. 2*E*, the autophosphorylation level of PKC is essentially the same throughout the range of final Ca^{2+} concentrations tested (0.01–1.0 mM), at both pH conditions. By contrast, the mole ratio of ANX7 phosphorylation is increased as the Ca^{2+} concentration increases. At pH 6.8, the Ca^{2+} titration curve for PKC-dependent ANX7 phosphorylation is biphasic. A minimal saturated phosphorylation level with a stoichiometry of 0.5 mol of P_i/mol of ANX7 is observed throughout the lower range of Ca^{2+} concentrations (0.01–0.20 mM), and this level is eventually increased as the free Ca^{2+} 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 Ca^{2+} concentration below 0.05 mM, and ANX7 phosphorylation eventually increases as the Ca^{2+} concentration increases beyond 0.05 mM. Thus, the action of Ca^{2+} 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 ^{33}P-labeled chromaffin cells. We also examined ANX7 phosphorylated *in vitro* by purified rat brain PKC. After carbachol or nicotine

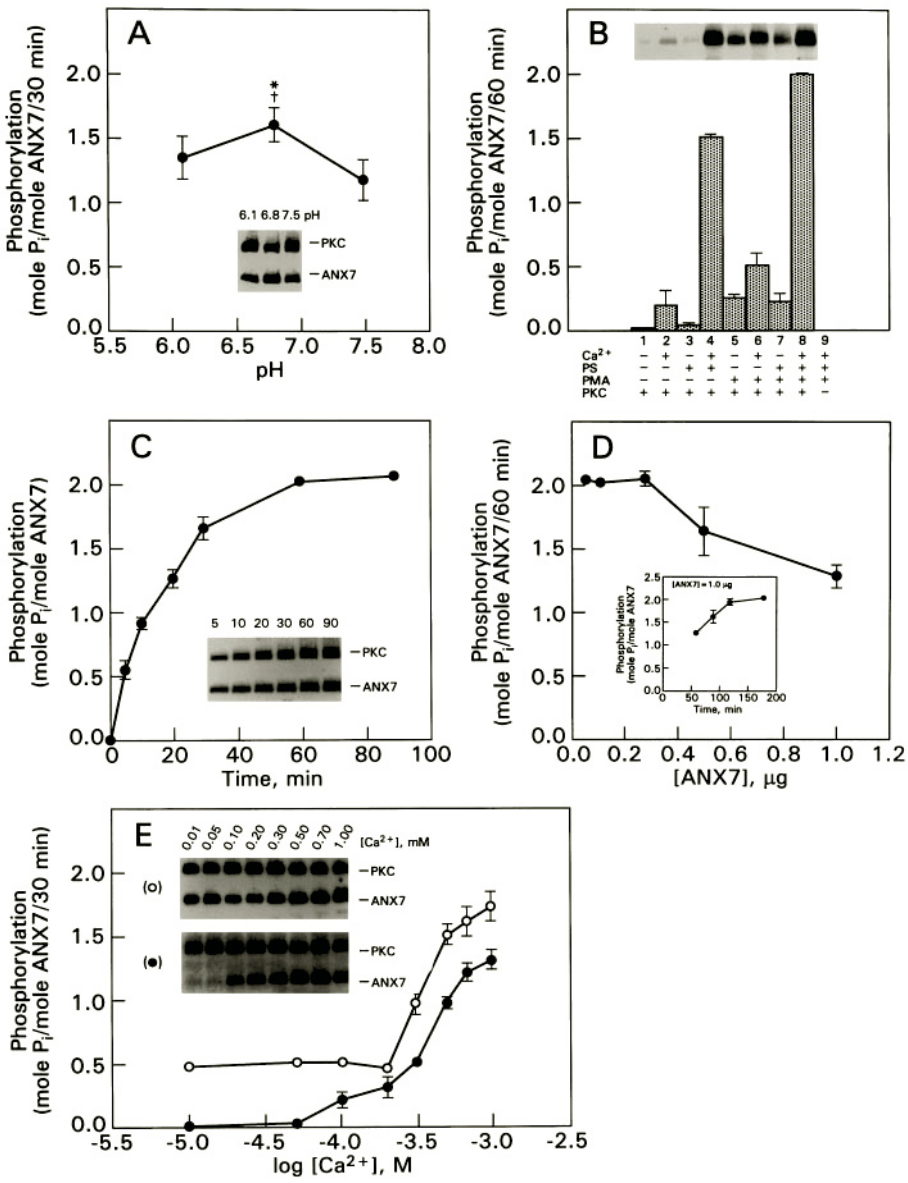

FIG. 2. ***In vitro* phosphorylation of ANX7 by protein kinase C.** *A*, pH dependence. ANX7 (0.25 μg) was incubated at 30 °C with 0.05 unit of PKC in 25 mM Tris-HCl (pH 7.5), 25 mM PIPES (pH 6.8), or 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and 100 μM [γ-^{33}P]ATP. *, $p <$ 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 $MgCl_2$, 100 μM [γ-^{33}P]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 $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, and 400 μg/ml PS liposomes. The reactions were stopped at the indicated time intervals after the addition of 100 μM [γ-^{33}P]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 Ca^{2+} 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.

stimulation, only labeled phosphoserine and phosphothreonine, but not phosphotyrosine, are detected in the immunoprecipitate (Fig. 3, *A* and *B*). Similarly, acid hydrolysis of ANX7 phosphorylated *in vitro* by PKC only yields labeled phosphoserine and phosphothreonine (Fig. 3*C*). These results thus support the hypothesis that phosphorylation of ANX7 in stimulated chromaffin cells is likely to be mediated by PKC.

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. 4*A*). Fig. 4*A* 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 Ca^{2+}, 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 in Fig. 4*B*, 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.

Ca^{2+} Dependence of Fusion Reactions Induced by Phosphorylated and Unphosphorylated ANX7—Because the fusion of lipid vesicles by ANX7 is dependent on Ca^{2+}, we then examined the effect of phosphorylation by PKC on the Ca^{2+} dependence of this fusion process. In these experiments, phosphorylated and unphosphorylated ANX7 were prepared as described above (see Fig. 4*A*), and their fusion activities were examined at different final Ca^{2+} concentrations, ranging from 0.01 to 1.0 mM. As shown in Fig. 5*A*, the rate of lipid vesicle fusion induced by phosphorylated ANX7 is markedly increased at lower Ca^{2+} concentrations, as compared with the same process induced by unphosphorylated ANX7 ($p < 0.005$). In addition, not only is

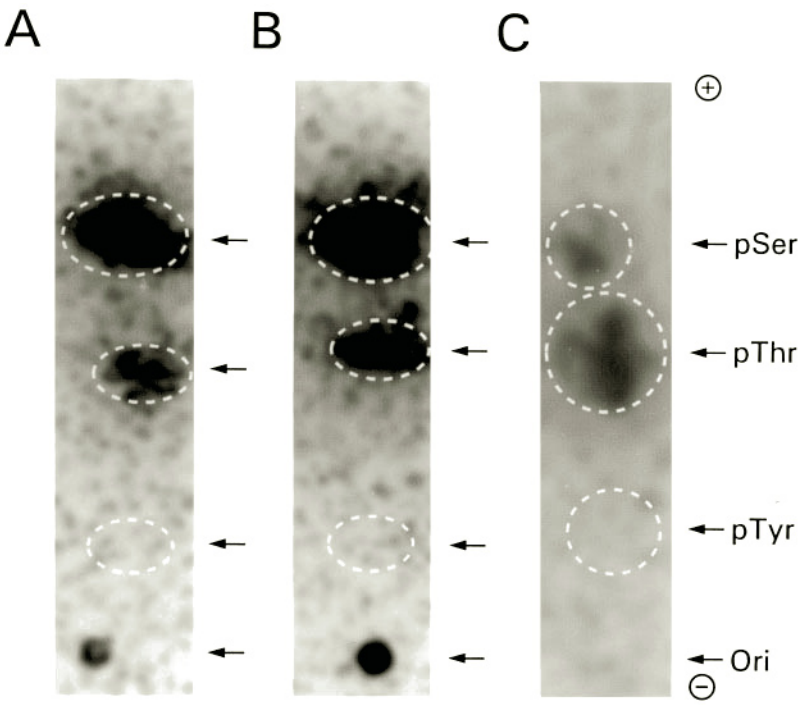

FIG. 3. **One-dimensional phosphoamino acid analysis of ANX7.** After autoradiography, the bands of ANX7 immunoprecipitated from stimulated chromaffin cells (*A* and *B*) or phosphorylated *in vitro* by PKC (*C*) were excised from the gel, and the phosphoamino acid analysis was carried out as described under "Experimental Procedures". *A*, stimulation with 100 μM carbachol. *B*, stimulation with 10 μM nicotine. *C*, *in vitro* phosphorylation by PKC. The positions of the standard phosphoamino acids are outlined by the *broken ovals*: phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), and phosphotyrosine (*P-Tyr*). *O* marks the position of the sample origin.

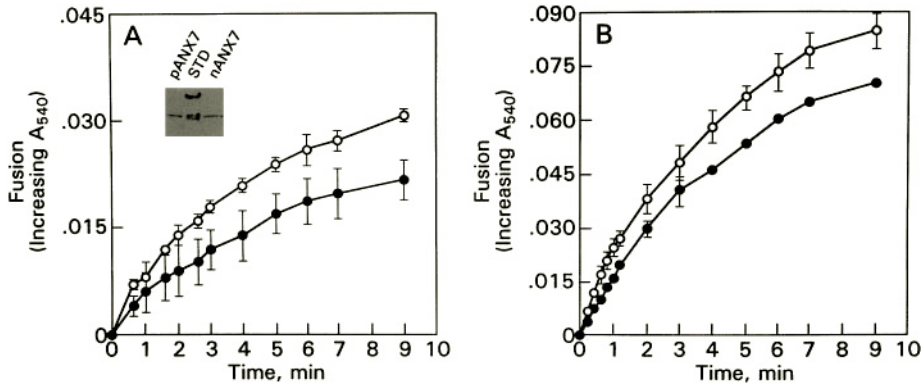

FIG. 4. **Effect of PKC-dependent phosphorylation on ANX7-induced lipid vesicle fusion.** *A*, phosphorylated (pANX7; *empty circles*) or unphosphorylated (nANX7; *filled circles*) ANX7 (0.5 μg), prepared as described under "Experimental Procedures," was added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 ml of lipid vesicle suspension. Fusion was initiated by the addition of 1 mM Ca^{2+} and measured by the change in absorbance at 540 nm after 20 min. The *inset* shows the equivalent amounts of the protein present in both reactions. *B*, ANX7 (1 μg) and PKC (0.5 unit) were added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 nM PMA, 100 μM ATP, and 0.5 ml of lipid vesicle suspension. The control was carried out in the absence of added ATP. Phosphorylation and fusion reactions were simultaneously initiated by the addition of 1 mM Ca^{2+} at room temperature. Fusion was measured by the change in absorbance at 540 nm after 30 min in the presence (*empty circles*) or absence (*filled circles*) of ATP. All data are the mean ± S.D. (n = 3).

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. 5*B*, the Ca^{2+} concentration required to induce half-maximal fusion activity (50% of F_{max}) is 200 μM 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 μM. Thus, Ca^{2+} not only potentiates the susceptibility of ANX7 to labeling by PKC (see Fig. 2*E*), 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 × *g*, and the protein bound to lipid vesicles was quantified by SDS-PAGE. The *inset* of Fig. 5*A* 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 $pp60^{c\text{-}src}$, each with an optimal enzymatic activity (Fig. 6*A*). Based on the calculation of ANX7 phosphorylation by these kinases, the amounts of phosphate

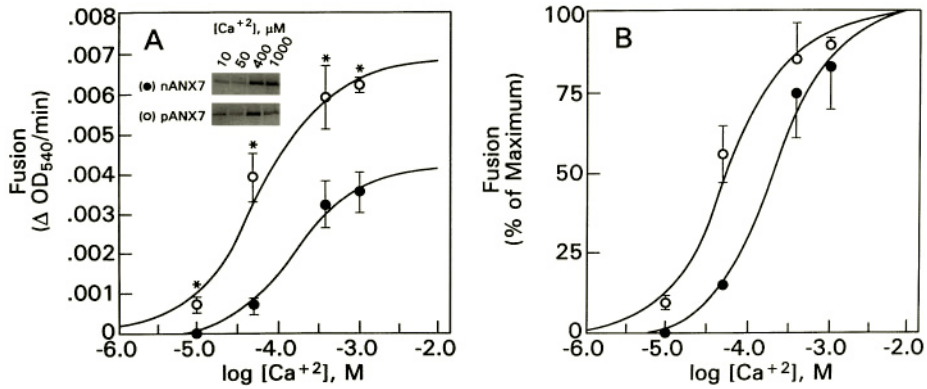

FIG. 5. **Ca^{2+} dependence of fusion of lipid vesicles by phosphorylated and unphosphorylated ANX7.** *A*, phosphorylated (*empty circles*) or unphosphorylated (*filled circles*) ANX7 (0.5 μg), prepared as described in Fig. 4*A*, was added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 ml of lipid vesicle suspension. Fusion was initiated by the addition of the indicated final Ca^{2+} 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 Ca^{2+} concentrations. *B*, data from *A* were replotted to highlight the increasing affinity of ANX7 for Ca^{2+} by PKC phosphorylation. The V_{max} of each curve was determined by a Lineweaver-Burk plot and then used as 100% maximal fusion activity. Based on these V_{max} values, the original data were transformed, expressed as a percentage of maximum of fusion activity, and replotted as shown.

incorporated into ANX7 were 0.7, 1.0, and 0.9 mol/mol of ANX7 when incubated for 60 min with PKG, PKA, and $pp60^{c\text{-}src}$, respectively (data not shown).

Based on these conditions, we then tested the consequences of phosphorylation by PKA, PKG, or $pp60^{c\text{-}src}$ for ANX7 activity on membrane fusion. Using the methods developed to study the PKC effect (see Fig. 4*B*), ANX7 was incubated in the presence of lipid vesicles and PKA, PKG plus cGMP, or $pp60^{c\text{-}src}$, with or without added ATP. As shown in Fig. 6*B*, 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 $pp60^{c\text{-}src}$ 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 Ca^{2+}-dependent membrane fusion reaction 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).

Ca^{2+} 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. 2*A*). 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.[2] 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 Ca^{2+}-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 Ca^{2+} modifies the conformation of ANX7 to permit enhanced labeling by PKC (see Fig. 2*E*). The evidence is that, although autophosphorylation of PKC remains unchanged, ANX7 phosphorylation is increased significantly as the free Ca^{2+} concentration elevates from 10 μM to 1 mM. The pH of the medium also dictates the pattern of Ca^{2+}-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 Ca^{2+} 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 Ca^{2+} concentration needed for ANX7-induced lipid vesicle fusion. PKC confers a $K_{½}$(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 Ca^{2+} concentration (*inset* of Fig. 5). Only the phosphorylated protein, however, is able to induce lipid vesicle fusion at lower Ca^{2+} concentrations (≤50 μM). At present, the mechanism by which the fusion activity of ANX7 is enhanced by PKC remains to be

[2] H. B. Pollard, unpublished data.

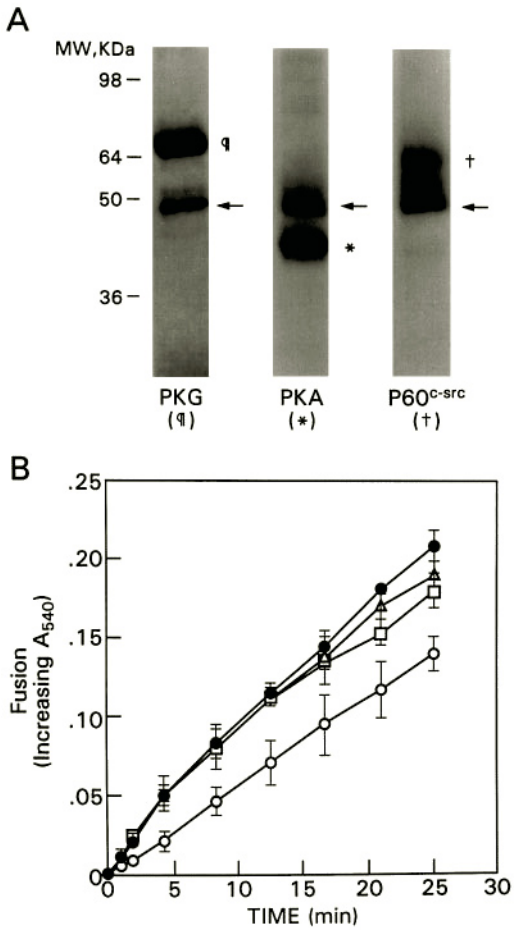

FIG. 6. ***In vitro* phosphorylation of ANX7 by various kinases and their effects on the fusion activity of ANX7.** *A*, ANX7 (0.25 μg) was incubated for 30 min at 30 °C with 200 units of PKG plus 10 μM cGMP, 50 units of catalytic subunit of PKA, or 10 units of pp60$^{c\text{-}src}$ in 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM [γ-^{33}P]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_{cat} (*), and pp60$^{c\text{-}src}$ (†). *B*, ANX7 (1 μg) and 2000 units of PKG plus 10 μM cGMP (*empty triangles*), 500 units PKA_{cat} (*empty squares*), or 100 units pp60$^{c\text{-}src}$ (*empty circles*) were added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 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^{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).

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^{2+} control site in the exocytotic machinery. In support of this hypothesis we recall that more than one factor may be involved in mediating the Ca^{2+} signal for exocytosis. For example, although synaptotagmin has been considered as a putative Ca^{2+} receptor for exocytosis (21), the knockout mutation of the synaptotagmin gene does not completely abolish Ca^{2+}-evoked secretion (57, 58). By contrast, the anx7 (−/−) knockout is lethal, whereas the anx7 (+/−) heterozygote expresses only low amounts of ANX7 protein and defectively secretes insulin (35). The lethality of the anx7 (−/−) nullizygous knockout thus serves to emphasize how critical ANX7 is to survival. The secretory defect of the heterozygote anx7 (+/−) animal serves to emphasize that the anx7 gene is critical for the secretory process in some tissues.

Other Kinases Phosphorylate ANX7 but Do Not Activate Membrane Fusion—Despite ANX7 phosphorylation occurring during a secretion-related phosphorylation event, we have not concluded that PKC is solely responsible for ANX7 phosphorylation in stimulated chromaffin cells. In this report, we show that ANX7 also serves as a good substrate for PKG, PKA, and pp60$^{c\text{-}src}$ *in vitro* (Fig. 6*A*). However, phosphorylation by these kinases either have no significant impact on ANX7-induced lipid vesicle fusion, as seen in the cases of PKA and PKG, or even decrease the rate of fusion of lipid vesicles by ANX7, as seen in the case of pp60$^{c\text{-}src}$. These results suggest that, in the presence of a secretion-related phosphorylation event, the fusion activity of ANX7 may be not activated by any of these kinases. This suggestion is supported by previously published reports. For example, in permeabilized chromaffin cells, cAMP (59, 60) and cGMP (60, 61) have little or no effect on Ca^{2+}-dependent catecholamine secretion. Thus, the direct involvement of PKA or PKG in exocytosis would appear to be ruled out. Similarly, pp60$^{c\text{-}src}$ appears not to be directly involved in exocytosis, because its activity is found to decrease following stimulation of the intact cell (62). Thus, although ANX7 is an efficient substrate for these kinases, we can only conclude that these kinases may be involved in regulating other as yet unidentified activities of ANX7. Recently, ANX7 has been shown to be phosphorylated by Src kinase, *in vitro* (63) and to be a tumor suppressor gene for prostate cancer (64).

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 involves lowering the $K_{½}$(app) for Ca^{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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