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MECHANISMS UNDERLYING $AlCl_3$ INHIBITION OF AGONIST-STIMULATED INOSITOL PHOSPHATE ACCUMULATION

ROLE OF CALCIUM, G-PROTEINS, PHOSPHOLIPASE C AND PROTEIN KINASE C*

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Abstract—Possible mechanisms of $AlCl_3$-induced inhibition of agonist-stimulated inositol phosphate (IP) accumulation were investigated using rat brain cortex slices, synaptosomes or homogenates. Under conditions in which $AlCl_3$ inhibits carbachol (CARB)-stimulated IP accumulation (G_p-mediated), $AlCl_3$ did not affect CARB (100 μM)-induced decreases (G_i-mediated) in 30 μM forskolin-stimulated cAMP accumulation, suggesting that $AlCl_3$ may be specific for G_p-mediated signal transduction. To determine whether $AlCl_3$ interfered with G_p function and/or phosphatidylinositol-specific phospholipase C (PiPLC) activity, effects of $AlCl_3$ on CARB- and Ca^{2+}-stimulated IP accumulation were examined in cortical synaptosomes. $AlCl_3$ (500 μM) decreased CARB (1 mM)- and Ca^{2+} (20 μM ionomycin)-stimulated IP accumulation to 77 and 75% of control, respectively, suggesting that $AlCl_3$ may not directly affect G_p activity, but does inhibit PiPLC activity. In cortical homogenates, $AlCl_3$ (10–500 μM) inhibited hydrolysis of [^{3}H]phosphatidylinositol 4,5-bisphosphate (PIP_2) by PiPLC in a concentration-dependent manner with an estimated IC_{50} of 100 μM. The effects of $AlCl_3$ on modulation of IP accumulation by extracellular Ca^{2+} and PKC were also examined as potential mechanisms. Decreasing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) from 1.0 to 0.1 mM decreased CARB-stimulated IP accumulation in slices. $AlCl_3$ (500 μM) decreased significantly 1 mM CARB-stimulated IP accumulation in 1.0 and 0.1 mM Ca^{2+} solutions; however, the effect of $AlCl_3$ on IP accumulation did not depend on $[Ca^{2+}]_e$. In cortical slices, inhibition of 1 mM CARB-stimulated IP accumulation by 500 μM $AlCl_3$ was not altered by the PKC activator phorbol 12,13-dibutyrate (PdBu, 1 μM), or the PKC inhibitor H-7 (10 μM), suggesting that $AlCl_3$ does not interfere with IP accumulation by activation of PKC. Other studies found that $AlCl_3$ (10–100 μM) inhibited PKC activity in a concentration-dependent manner in both cytosolic and membrane fractions of cortical homogenates with an estimated IC_{50} of 60 μM. These results support the hypothesis that $AlCl_3$ inhibition of agonist-stimulated IP accumulation may be mediated by inhibition of PiPLC activity, rather than disruption of G-protein function or modulation of the IP signalling system by Ca^{2+} or PKC.

Key words: aluminum; phosphoinositide hydrolysis; protein kinase C; cAMP; phospholipase C; brain slices

Aluminum-induced neurotoxicity has been demonstrated in humans following occupational exposure [1, 2] and following renal dialysis with aluminum-contaminated solutions [3]. Clinical symptoms of the dialysis syndrome include loss of coordination, difficulty in speaking, and loss of memory and are accompanied by neuropathological changes in the central nervous system. Recently, decreases in cognitive function have been correlated with body aluminum burden in dialysis patients not exhibiting clinical symptoms of the dialysis syndrome [4]. Aluminum also impairs cognitive function in animals [5, 6]. Elevated levels of aluminum in the drinking water of rats impaired consolidation and extinction of passive avoidance tasks [6]. The mechanism(s) underlying aluminum-induced neurotoxicity has not been clearly elucidated.

Recent studies have indicated that, under conditions in which $AlCl_3$ alters passive avoidance behavior [6], significant neurochemical alterations occur in the central nervous system. These changes

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include reduced IP* levels in the hippocampus [7] and increased distribution of PKC to the membrane [8]. *In vitro* studies have demonstrated that $AlCl_3$ decreased CARB-stimulated IP accumulation in rat brain slices [9]. We recently confirmed and extended the observation of Johnson and Jope [9] by demonstrating that $AlCl_3$ produces concentration-dependent decreases in IP accumulation stimulated by activation of muscarinic, adrenergic and metabotropic glutamate receptors in cortical and hippocampal brain slices [10]. $AlCl_3$ also inhibits IP accumulation stimulated by direct activation of G-proteins with NaF [10, 11]. The inhibition by $AlCl_3$ of IP accumulation stimulated by receptor and G-protein activation suggest that $AlCl_3$ may interfere with IP accumulation by acting downstream of receptor activation or receptor–G protein interaction [10].

We sought to characterize further mechanisms by which $AlCl_3$ interferes with agonist-stimulated IP accumulation in rat brain slices, including effects on G-protein function and phospholipase C (PiPLC) activity. Other data have also suggested that $AlCl_3$ alters PKC activity [8, 12] and interferes with Ca^{2+} binding to the membrane [13], Ca^{2+} entry [14] and Ca^{2+} extrusion [15, 16]. Since extracellular Ca^{2+} and PKC can modulate IP accumulation [for review see Ref. 17], we also investigated whether $AlCl_3$ inhibits IP accumulation via interaction with Ca^{2+} and/or PKC. The results of the present experiments indicate that $AlCl_3$ interferes with IP accumulation by inhibiting PiPLC activity.

MATERIALS AND METHODS

Solutions. KRB solution contained (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; KH_2PO_4, 1.18; $CaCl_2$, 0.75; D-glucose, 10 (pH 7.4). HEPES buffer solution contained (mM): NaCl, 122; KCl, 4.9; $MgSO_4$, 1.2; $NaHCO_3$, 3.6; $CaCl_2$, 0.75; HEPES, 30; and D-glucose, 11 (pH 7.4). During incubation of slices to measure IP accumulation, buffer solutions contained 10 mM LiCl. Acidified chloroform/methanol solution contained 50:100:1 chloroform:methanol:HCl and was prepared prior to each experiment. *myo*-[^{3}H]Inositol, [^{3}H]adenine, [γ-^{32}P]ATP and [^{3}H]PIP_2 were purchased from DuPont/New England Nuclear (Boston, MA). $AlCl_3$ was purchased from the Sigma Chemical Co. (St. Louis, MO). Stock solutions of $AlCl_3$ were made daily by dissolving it in deionized water and diluting to the necessary concentration in buffer solutions. The pH of KRB and HEPES buffer solutions was not affected by the addition of $AlCl_3$. All other chemicals were reagent grade or the highest available grade and were purchased from commercial vendors.

PI hydrolysis assay. Slices (350 × 350 μm) from the frontal cortex of adult male Long–Evans rats (250–300 g) were made by chopping the cortex in perpendicular directions using a McIlwain tissue chopper. Slices were washed three times by incubation at 35° in oxygenated KRB buffer solution (unless otherwise noted) for 10 min in a shaking water bath. Inositol phospholipids were labeled by incubation with 3.2 μM *myo*-[^{3}H]inositol (15 Ci/mmol) in oxygenated KRB buffer solution for 90 min with continuous shaking. Prior to adding the *myo*-[^{3}H]inositol, it was purified by passing over a 300-μL bed of Dowex ion exchange resin. Following incubation, excess *myo*-[^{3}H]inositol was removed by washing slices three times with 5 mL of KRB buffer solution.

To measure agonist-stimulated accumulation of [^{3}H]IPs, 50 μL of slices (approximately 0.25 mg) was added to 200 μL of KRB buffer solution containing $AlCl_3$ and allowed to incubate for 30 min. IP accumulation was then stimulated by the addition of 10 μL of KRB buffer solution containing the appropriate concentration of agonist. Agonist concentrations given in Results refer to the final concentration of agonist (and/or $AlCl_3$) in KRB buffer solution. After 30 min of incubation at 35°, stimulation of slices was terminated by the addition of 1 mL of ice-cold acidified chloroform/methanol solution.

To quantify IPs, the method of Berridge *et al.* [18] was used with some modifications. Aqueous IPs and organic phosphoinositides were separated by the addition of 0.4 mL of chloroform and 0.4 mL of deionized water, followed by vortexing for 5 sec. A 0.8-mL aliquot of the aqueous phase was diluted with deionized water to 3.3 mL, 2 mL of Dowex AG 1-X8 (formate form) slurry was added, and the solution was placed in a BioRad® column. Columns were rinsed with 5 mL of deionized water, followed by 8 mL of 5 mM *myo*-inositol, and then 8 mL of 5 mM sodium formate/60 mM sodium tetraborate solution. IPs were eluted by two 4-mL aliquots of 1.4 M ammonium formate/0.1 M formic acid. A 2-mL aliquot of the eluted IPs was diluted with 2 mL of deionized water to prevent precipitation of salts upon addition of 10 mL of Aquasol II® scintallation fluor. The incorporation of *myo*-[^{3}H]inositol into phosphoinositides was measured by taking a 50-μL aliquot of the organic layer, evaporating to dryness, adding liquid scintallation fluid and counting. A Beckman liquid scintillation counter with internal quench curves was used for counting of both the aqueous and organic samples. Total [^{3}H]IP accumulation was calculated as dpm aqueous phase/total dpm incorporated into lipids (dpm aqueous + dpm organic). In a typical experiment, the incorporation of [^{3}H]inositol into lipids is approximately 50,000 dpm/tube. The median value of triplicate measurements was used to calculate mean ± SEM values shown in Results.

Cyclic AMP accumulation. Agonist-stimulated cAMP accumulation was measured in cortical slices under conditions identical to those used to measure IP accumulation. Cortical slices were prepared using the procedure described above, and ATP pools were labeled by incubating in KRB buffer solution containing 0.775 μM [^{3}H]adenine (25.8 Ci/mmol) for 1 hr. All solutions used after incorporation contained 1 mM isobutyl methylxanthine. Following a 30-min preincubation with $AlCl_3$, slices were stimulated

* Abbreviations: CARB, carbachol; PiPLC, phosphatidylinositol-specific phospholipase C; PKC, protein kinase C; PdBu, phorbol 12,13-dibutyrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol phosphate(s); and KRB, Krebs–Ringer buffer.

with forskolin for 15 min. Forskolin was dissolved in dimethyl sulfoxide at a high concentration and diluted to the necessary concentrations in buffer solution (<1% dimethyl sulfoxide, which did not affect cAMP accumulation). Carbachol (100 μM) was added simultaneously with forskolin. The accumulation of [^{3}H]cAMP was stopped by the addition of 50 μL of 1.3 N HCl, and samples were placed immediately on ice.

To quantitate formation of [^{3}H]cAMP, the method of Alvarez and Daniels [19] was used. Slices were centrifuged for 10 min at 500 *g* and a 200-μL aliquot of the supernatant was removed and added directly to columns containing 1.3 to 1.5 g acidic alumina. The remaining slices and supernatant were sonicated and protein content was assayed using the method of Bradford [20]. Columns were washed twice with 4 mL of 0.005 N HCl solution followed by 1 mL of 0.1 M ammonium acetate solution. [^{3}H]cAMP was eluted from the column by the addition of 3.5 mL of 0.1 M ammonium acetate solution and collected in scintillation vials. Radioactive content was determined by counting in 10 mL of Ultima Gold scintillation fluid in a Beckman scintillation counter. Results are expressed as dpm [^{3}H]cAMP formed/mg protein, and mean ± SEM values were calculated from the average value of triplicate measurements. The content of [^{3}H]cAMP in slices measured at the conclusion of incorporation was subtracted from all samples.

Protein kinase C activity. Cytosolic and membrane fractions of cortical homogenates were prepared using the method of Malkinson *et al.* [21]. Briefly, tissue was homogenized (100 mg/mL) in ice-cold homogenizing buffer containing (mM): Tris–HCl, 20; EDTA, 2; β-mercaptoethanol, 50; phenylmethylsulfonyl fluoride, 0.5; and sucrose, 320 (pH 7.1). The homogenate was centrifuged for 30 min at 100,000 *g* in a Ti50 rotor using a Beckman L8-70 ultracentrifuge. The supernatant was held in reserve and the pellet was resuspended (100 mg/mL) in homogenizing buffer and the centrifugation step was repeated. The supernatants were combined to make up the cytosolic fraction and the pellet was resuspended to approximately 200 mg/mL in homogenizing buffer containing 0.2% Triton X-100, sonicated, and incubated on ice for 30 min. The homogenate was then centrifuged at 100,000 *g* for 30 min and the resulting supernatant was saved as the membrane fraction. The protein content of each fraction was determined using the Bradford [20] assay.

Protein kinase C activity was determined as described by Govoni *et al.* [22] by measuring the incorporation of ^{32}P into histone from [γ-^{32}P]ATP (10 Ci/mmol). To measure stimulated activity, 10 μg of soluble or membrane protein was added to solution containing 20 mM Tris–HCl, 5 mM $MgCl_2$, 500 μg/mL histone (type IIIs), 2 mM $CaCl_2$ and 40 μg/mL phosphatidylserine (pH 7.5). To measure unstimulated activity of protein kinases, $CaCl_2$ and phosphatidylserine were replaced by 2 mM EGTA. $AlCl_3$ was dissolved in water and added to the solutions at the required concentrations. If necessary, the pH of the solution was re-adjusted to pH 7.5 following addition of $AlCl_3$. Following a 5-min preincubation in a 35° water bath, the assay was initiated by the addition of 5 nmol/sample of ATP containing 1 μCi[γ-^{32}-P]ATP. The final volume of each sample was 250 μL. After 5 min the assay was terminated by the addition of 1 mL of ice-cold 25% trichloroacetic acid and placing samples on ice. Each sample was filtered onto a 0.45-μm membrane filter, and the sample tube was rinsed with 1 mL of trichloroacetic acid solution. Filters were then washed twice with 2 mL of trichloroacetic acid solution and placed into a scintillation vial. Ten milliliters of Ultima Gold was added and samples were counted in a Beckman scintillation counter. Values from triplicate measurements were averaged, and PKC activity was determined from the net difference between unstimulated activity and Ca^{2+}/phosphatidylserine-stimulated activity. Results are expressed as nmol/min/mg protein.

Synaptosomal IP accumulation. Synaptosomes were prepared by the method of Dodd *et al.* [23]. The frontal cortex was homogenized in 0.32 M sucrose and centrifuged at 1000 *g* for 10 min. The supernatant was layered over 1.2 M sucrose and centrifuged in the Beckman Ultracentrifuge at 150,000 *g* for 25 min in a Ti70 rotor. The synaptosomal/myelin fraction was collected from the interface and diluted in 0.32 M sucrose, then layered over 0.8 M sucrose and centrifuged as above. The synaptosomal pellet was resuspended in HEPES-buffer solution containing 3.2 μM *myo*-[^{3}H]inositol. Synaptosomes were incubated in a 35° shaking water bath for 60 min to allow the incorporation of *myo*-[^{3}H]inositol into inositol phospholipids. Following incorporation, synaptosomes were washed three times with 4 mL of HEPES buffer solution by centrifugation at 15,000 *g* for 10 min followed by resuspension in fresh buffer solution. Hydrolysis of phosphoinositides was measured as described above for cortical slices.

Phospholipase C activity. PiPLC activity in homogenates of frontal cortex was measured using the method of Bergers *et al.* [24] with modifications. Frontal cortex of rat brain was homogenized at approximately 4 mg/mL in buffer containing 20 mM Tris base, 1 mM $CaCl_2$ and 100 mM KCl, pH 7.4. A stock solution of $AlCl_3$ (20 mM) was prepared in this buffer solution, the necessary dilutions were made, and the pH was re-adjusted to 7.4, if necessary. Preliminary experiments were conducted to determine optimal assay conditions with respect to substrate concentration and to ensure that the assay was linear with respect to time and tissue concentration. To determine the effects of $AlCl_3$ on PiPLC activity, 5 μL of homogenate was added to 5 μL of buffer ± $AlCl_3$ and incubated in a 35° water bath for 3–5 min, followed by the addition of 10 μL of substrate solution containing 300 nmol/mL PIP_2, 0.4 mg/mL cetrimide and 1.5 μCi/mL [^{3}H]PIP_2 (8.8 Ci/mmol). After 7 min of incubation, the reaction was stopped and the lipid portion extracted by the addition of 500 μL of chloroform : methanol (1 : 1) solution and 500 μL of 1 N HCl. The samples were vortexed thoroughly, followed by centrifugation at 1000 *g* for 10 min. Inositol phosphate accumulation in the aqueous phase was determined by taking a 200-μL aliquot and drying under N_2 for 15 min,

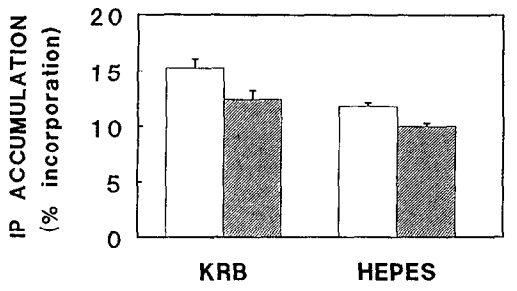

Fig. 1. Effect of phosphate-free buffer solutions on inhibition of IP accumulation by $AlCl_3$. The effect of $AlCl_3$ on 1 mM CARB-stimulated IP accumulation was examined in phosphate-containing Krebs–Ringer buffer (KRB) and phosphate-free HEPES buffer solution (HEPES). IP accumulation in the absence (open bars) and presence (striped bars) of 500 μM $AlCl_3$ is shown. Control basal IP accumulation was 8.3 ± 0.7 and 6.9 ± 0.4% of incorporation in KRB and HEPES buffer solutions, respectively. Two-way ANOVA indicated a significant main effect of 500 μM $AlCl_3$ as well as a significant main effect of buffer solution ($P < 0.05$), but no interaction. Results are the means ± SEM of five separate experiments, where each experimental value is the median of triplicate measurements.

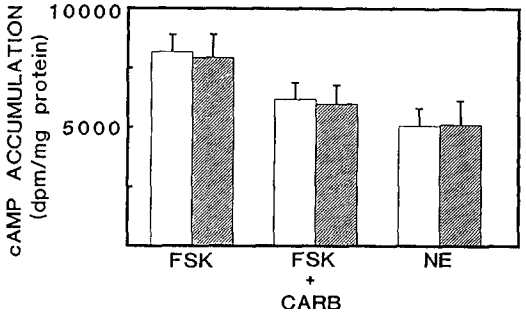

Fig. 2. Effect of $AlCl_3$ on cAMP formation in cortical slices. Cortical slices were incubated in the presence of 30 μM forskolin (FSK) or 100 μM norepinephrine (NE) for 15 min to measure direct or receptor-stimulated activation of adenylate cyclase, respectively. The effect of 500 μM $AlCl_3$ (striped bars) on inhibition of adenylate cyclase by 100 μM CARB was examined by preincubating slices with $AlCl_3$ for 30 min, followed by a 15-min stimulation with forskolin. Control basal cAMP accumulation was 1400 ± 550 dpm/mg protein. Two-way ANOVA indicated a significant ($P < 0.05$) main effect of CARB on forskolin-stimulated cAMP accumulation. Results are the means ± SEM of five separate experiments, where each experimental value is the average of triplicate measurements.

followed by counting in a scintillation counter in 5 mL of Ultima Gold scintillation fluid. Accumulation of ^3H in the aqueous component was used to calculate pmol of PIP_2 hydrolyzed/min/mg protein [20] after subtraction of a hydrolysis blank. The median of triplicate measurements was used to calculate mean ± SEM values shown in Results.

RESULTS

Effects of phosphate on the inhibition of IP accumulation by $AlCl_3$. We have reported previously that aluminum inhibits basal and agonist-stimulated IP accumulation in brain slices incubated in phosphate-containing buffer solutions and that the ability of aluminum to interact with IP accumulation depends on the anionic salt of aluminum used [10]. Others have reported that $AlCl_3$ blocks CARB-stimulated IP accumulation in slices incubated in phosphate-free buffer solutions [9]. Phosphate is often excluded from buffer solutions when aluminum effects are examined *in vitro* due to the potential formation of insoluble aluminum phosphate. Prior to examining mechanisms of $AlCl_3$ action, we sought to determine if the omission or inclusion of phosphate in buffer solutions influenced $AlCl_3$ inhibition of CARB-stimulated IP accumulation. Effects of phosphate on the inhibition of CARB-stimulated IP formation by $AlCl_3$ were examined by measuring IP accumulation in cortical slices prepared in phosphate-containing KRB buffer solution or phosphate-free HEPES buffer solution. In this experiment, the frontal cortex from each rat was divided in half, sliced, and placed in KRB or HEPES buffer solution. CARB (1 mM)-stimulated IP accumulation was significantly lower in slices prepared in HEPES buffer solution than in slices prepared in KRB buffer solution (Fig. 1). Addition of 500 μM $AlCl_3$ significantly reduced CARB-stimulated IP accumulation in both KRB and HEPES buffer solutions when compared with their respective control values (Fig. 1). There was no significant interaction between $AlCl_3$ and buffer solution. Similar results were obtained using KRB buffer solution in which the KH_2PO_4 was omitted (results not shown). Since IP accumulation was more robust in KRB buffer solution, we continued to use this buffer for other experiments.

Effects of $AlCl_3$ *on G-proteins in slices.* $AlCl_3$ may inhibit agonist-stimulated IP accumulation in slices by disrupting the function of G-proteins [10]. Aluminum inhibits the GTPase activity of transducin [25, 26] and tubulin [27] and interferes with other G-protein-mediated processes including IP accumulation [9–11, 28, 29]. Since it is possible that $AlCl_3$ may interfere with G-protein activity in a manner that is not subtype selective, we examined the effect of $AlCl_3$ on muscarinic inhibition of forskolin-stimulated cAMP formation under conditions nearly identical to those used previously to measure IP accumulation [10]. Basal cAMP accumulation was not affected by 500 μM $AlCl_3$. CARB (100 μM) significantly inhibited cAMP accumulation stimulated by 30 μM forskolin in cortical slices (Fig. 2). There was no significant effect of 500 μM $AlCl_3$ on forskolin-stimulated cAMP formation or CARB-mediated inhibition of cAMP formation in cortical slices. Norepinephrine (100 μM) stimulated cAMP formation, but the norepinephrine-stimulated cAMP accumulation was also not affected by 500 μM $AlCl_3$ (Fig. 2).

Effects of $AlCl_3$ *on PiPLC activity.* $AlCl_3$-induced inhibition of agonist-stimulated IP accumulation may be due to a direct effect on PiPLC activity. Aluminum

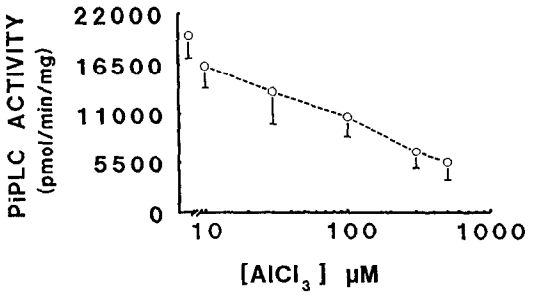

Fig. 3. Inhibition of PIP_2 hydrolysis by $AlCl_3$. Hydrolysis of [^{3}H]PIP_2 by PiPLC was examined in the presence of increasing concentrations of $AlCl_3$ in cortical homogenates. Approximately 20 μg of protein was added to solution containing the indicated concentrations of $AlCl_3$, and reaction mixture containing 300 nmol/mL PIP_2, 1.5 μCi/mL [^{3}H]PIP_2 and 0.4 mg/mL cetrimide was added to initiate the reaction. After 7 min, the reaction was stopped and inositol phosphate accumulation was determined as described in Materials and Methods. Results are the means ± SEM of five separate experiments, where each experimental value is the median of triplicate measurements. One-way ANOVA followed by Fisher's least significant difference (lsd) test indicated a significant effect ($P < 0.05$) of 100 μM and greater $AlCl_3$ concentrations on PiPLC activity when compared with control.

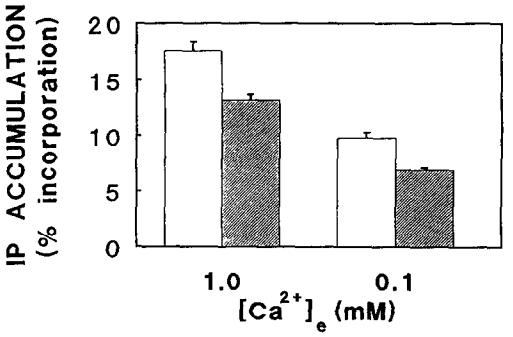

Fig. 4. Effect of extracellular Ca^{2+} on $AlCl_3$ inhibition of CARB-stimulated IP formation. The effect of $[Ca^{2+}]_e$ on inhibition of CARB-stimulated IP accumulation was examined in cortical slices. Slices were incubated in KRB solution containing 1.0 or 0.1 mM Ca^{2+} in the absence (open bars) or presence (striped bars) of 500 μM $AlCl_3$ and were stimulated with 1 mM CARB. Results are the means ± SEM of six experiments, where each experimental value is the median of triplicate measurements. Two-way ANOVA indicated significant main effects ($P < 0.05$) of $AlCl_3$ and $[Ca^{2+}]_e$, but no interactions. Control basal IP accumulation was 8.1 ± 0.6 and 4.6 ± 0.2% of incorporation in 1.0 and 0.1 mM Ca^{2+} solutions, respectively.

inhibits PIP_2 hydrolysis by PiPLC isolated from bovine heart [30], and indirect effects of $AlCl_3$ on PiPLC have also been reported in mouse neuroblastoma cells [29]. Depolarization-induced influx of Ca^{2+} has been used to stimulate PiPLC and IP accumulation in slices [31, 32]. However, $AlCl_3$ may block depolarization-induced Ca^{2+} influx [14] potentially confounding the interpretation of effects of $AlCl_3$ on IP accumulation stimulated by this method. Ca^{2+} stimulation of PiPLC has also been demonstrated in synaptosomes following permeabilization with Ca^{2+} ionophores [33] and is a viable method by which to examine the effects of $AlCl_3$ on PiPLC. Therefore, to determine whether the inhibition of IP accumulation by $AlCl_3$ is the result of a decrease in PiPLC activity, we examined the effect of $AlCl_3$ on IP accumulation in synaptosomes stimulated by CARB or the Ca^{2+} ionophore ionomycin. $AlCl_3$ (500 μM) decreased both CARB (1 mM)- and ionomycin (20 μM)-stimulated IP accumulation to 77 ± 12 and 75 ± 13% of control (N = 4), respectively, in cortical synaptosomes. In separate experiments, 500 μM $AlCl_3$ did not decrease $^{45}Ca^{2+}$ entry into synaptosomes in the presence of ionomycin (data not shown), suggesting that $AlCl_3$ interferes directly with PiPLC activity. To confirm this, we investigated whether $AlCl_3$ would inhibit brain PiPLC activity. Figure 3 shows that $AlCl_3$ (10–500 μM) inhibited hydrolysis of [^{3}H]PIP_2 in a concentration-dependent manner with an estimated IC_{50} of 100 μM. $AlCl_3$ concentrations of 100 μM and greater significantly inhibited PiPLC activity in cortical homogenates.

Interactions of $AlCl_3$ *with extracellular* Ca^{2+}. It has been reported that the concentration of extracellular Ca^{2+} modulates CARB-stimulated IP accumulation in slices [33–37]. Aluminum has been reported to displace Ca^{2+} from binding sites on artificial membranes [13] and block influx of $^{45}Ca^{2+}$ into synaptosomes [14]. Therefore, $AlCl_3$ may decrease CARB-stimulated IP accumulation by decreasing the availability of Ca^{2+} to modulate IP accumulation. To examine the potential role of competition between $AlCl_3$ and extracellular Ca^{2+} concentration on IP accumulation, slices were prepared and [^{3}H]inositol was incorporated in Ca^{2+}-free KRB buffer solution. Following incorporation, the slices were washed in KRB solution containing 1 or 0.1 mM Ca^{2+}, and subsequent exposure to $AlCl_3$ (500 μM) and 1 mM CARB was carried out in each solution. The inhibition of CARB-stimulated IP accumulation by $AlCl_3$ was not altered by the 10-fold reduction in the extracellular Ca^{2+} concentration (Fig. 4).

Effects of $AlCl_3$ *on PKC activity.* Activation of PKC has been shown to decrease phosphoinositide hydrolysis in neuronal tissue [38–40], including hydrolysis stimulated by CARB [35, 41, 42]. *In vivo* exposure to $AlCl_3$ increases phosphorylation of brain cytoskeletal proteins [43] and increases the distribution of PKC to the membrane fraction [8], suggesting that PKC may be activated in $AlCl_3$-exposed animals. Therefore, we examined in greater detail the effects of $AlCl_3$ on PKC. In both the cytosolic and membrane fractions of cortical homogenates, $AlCl_3$ inhibited PKC activity in a concentration-dependent manner (Fig. 5). At 30 μM $AlCl_3$, PKC activity was 72.9 ± 16.3% (N = 5) and 88.4 ± 12.4% (N = 6) of control in cytosolic and membrane fractions, respectively. At 100 μM $AlCl_3$, the highest concentration tested, PKC activity was 23.3 ± 10.7% (N = 6) and 21.2 ± 13.5% (N = 6) of

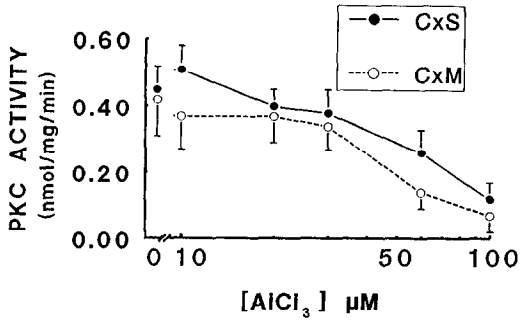

Fig. 5. Effects of $AlCl_3$ on PKC activity in cortical homogenates. PKC activity in the membrane (○) and soluble (●) components of cortical homogenates was measured in the presence of increasing concentrations of $AlCl_3$. PKC activity was measured by the incorporation of ^{32}P from ATP into histone in the presence of Ca^{2+} and phosphatidylserine. Phosphorylation in the absence of phosphatidylserine and Ca^{2+} has been subtracted from these values. One-way ANOVA indicated a significant effect of $AlCl_3$ and post-hoc analysis using Fisher's lsd test indicated that 100 μM $AlCl_3$ was significantly different from control in the soluble fraction and 60 and 100 μM $AlCl_3$ were significantly different from control in the membrane fraction ($P < 0.05$). Results are the means ± SEM of five or six separate experiments where each experimental value is the average of triplicate measurements.

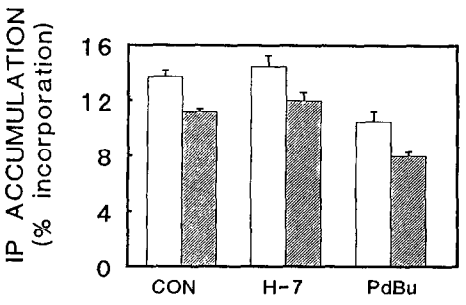

Fig. 6. Lack of interaction of $AlCl_3$ with activators and inhibitors of protein kinase C. Effects of the protein kinase C inhibitor H-7 (10 μM) and the activator phorbol 12,13-dibutyrate (PdBu, 1 μM) on 1 mM CARB-stimulated IP accumulation in cortical slices were examined in the absence (open bars) and presence (striped bars) of 500 μM $AlCl_3$. Basal IP accumulation was 6.6 ± 0.2% of incorporation. Results shown are the means ± SEM of three separate experiments, where each experimental value is the median of triplicate measurements. Two-way ANOVA indicated a significant main effect of 500 μM $AlCl_3$ treatment and of PdBu treatment ($P < 0.05$). There was no interaction between $AlCl_3$ and PdBu or H-7.

control in the cytosolic and membrane fractions, respectively. The estimated IC_{50} for $AlCl_3$ inhibition was approximately 60 μM. Other experiments examined the effects of $AlCl_3$ on CARB-stimulated IP accumulation in the presence of a PKC activator or inhibitor. If $AlCl_3$ inhibits CARB-stimulated IP accumulation by a PKC-mediated mechanism, it should interact with known activators or inhibitors of PKC. Effects of $AlCl_3$ on CARB-stimulated IP accumulation in the presence of H-7, a PKC inhibitor, and PdBu, a PKC activator, are shown in Fig. 6. In the absence of $AlCl_3$, 10 μM H-7 produced a slight increase in CARB-stimulated IP accumulation (Fig. 6). By contrast, 1 μM PdBu significantly decreased CARB-stimulated IP accumulation. $AlCl_3$ (500 μM) significantly decreased CARB-stimulated IP accumulation under all treatment conditions. There was no interaction between $AlCl_3$ and H-7 or PdBu.

DISCUSSION

These experiments were designed to determine the potential site(s) of action underlying $AlCl_3$ inhibition of agonist-stimulated IP accumulation in brain slices. The results indicate that $AlCl_3$ inhibition of IP accumulation is not influenced by $[Ca^{2+}]_e$, nor by changes in phosphate composition of buffer solutions. Furthermore, the effects of $AlCl_3$ on IP accumulation in slices are not the result of activation of PKC by $AlCl_3$. Under the conditions of these studies, $AlCl_3$ did not affect inhibition of cAMP formation by CARB, suggesting that $AlCl_3$ has no effect on signal transduction via G_i. That $AlCl_3$ decreased hydrolysis of PIP_2 and inhibited Ca^{2+}-stimulated IP accumulation in synaptosomes suggests that an effect of $AlCl_3$ on PiPLC activity may be responsible for the inhibition of agonist-stimulated IP accumulation in slices.

Aluminum ion in aqueous solution has complex chemistry which is dependent on pH and anionic species composition [44]. The toxicity of aluminum both *in vivo* [45, 46] and *in vitro* [47, 48] is influenced by or dependent on the anion(s) present. Previous results from our laboratory have indicated that $AlCl_3$ and aluminum lactate inhibit CARB-stimulated IP accumulation more effectively than aluminum citrate [10]. Effects of $AlCl_3$ on IP accumulation had been reported in phosphate-free [9] and phosphate-containing [10] buffer solutions, but the influence of phosphate on $AlCl_3$ inhibition of IP accumulation had not been examined systematically. The results presented here demonstrate that $AlCl_3$ affects IP accumulation similarly in phosphate-containing and phosphate-free solutions. These results are important with respect to the ability of aluminum to decrease IP content in the hippocampus when administered in drinking water [7], since phosphate concentrations in blood and body fluids are near 2 mM. The ability of $AlCl_3$ to decrease IP accumulation *in vitro* in the presence of phosphate supports the possibility that aluminum may have a direct effect on inositol phosphate systems *in vivo*.

The results of the present experiments suggest that $AlCl_3$-induced inhibition of agonist-stimulated IP accumulation may be the result of $AlCl_3$ effects on PiPLC activity. $AlCl_3$ inhibits IP accumulation stimulated by CARB [9, 10], norepinephrine, quisqualate [10] and bradykinin [28] as well as IP accumulation stimulated by NaF [9, 10] and GTPγS [29]. These observations suggest that $AlCl_3$ may

interfere with G_p, PiPLC, or both. We used CARB and ionomycin to separate IP accumulation stimulated by receptor–G-protein complex activation from direct activation of PiPLC by Ca^{2+}, respectively. Results from these experiments suggested that PiPLC is a critical site of $AlCl_3$ action during the inhibition of agonist-stimulated IP accumulation, as $AlCl_3$ inhibited CARB- and ionomycin-stimulated IP accumulation to the same extent (approximately 25%). Recently, Shi *et al.* [29] reported that $AlCl_3$ inhibits G-protein- and Ca^{2+}-stimulated inositol phosphate accumulation in neuroblastoma cells, which suggested to them that $AlCl_3$ affected PiPLC activity. We report in this study that $AlCl_3$ inhibits unstimulated PiPLC activity in cortical homogenates in a concentration-dependent manner at concentrations similar to those that inhibit basal and agonist-stimulated IP accumulation [10]. Although it has been reported previously that $AlCl_3$ inhibits PiPLC activity isolated from heart tissue [30], our data are the first to show a direct effect of $AlCl_3$ on brain PiPLC.

While our data do not allow us to exclude completely the possibility that $AlCl_3$ acts on G_p [29], the effects of $AlCl_3$ on PiPLC activity may be sufficient to account for $AlCl_3$ effects on IP accumulation. This is supported by several observations. Effects of $AlCl_3$ on IP accumulation are generally small in magnitude. In our studies in slices, we observed a 25% reduction in CARB-stimulated IP accumulation in the presence of 500 μM $AlCl_3$ [10]. This is similar to the 15% decrease in IP_3 content in the hippocampus of rats exposed to aluminum in the drinking water [7] and to the 25% reduction in IP accumulation in the presence of 500 μM $AlCl_3$ observed in synaptosomes in this study. Since CARB-stimulated IP accumulation is the result of G_p activation, and Ca^{2+}-stimulated IP accumulation is the result of direct activation of PiPLC, one might have expected no effect on the latter if $AlCl_3$ acted only on G_p, or greater effects of $AlCl_3$ on CARB-stimulated versus Ca^{2+}-stimulated IP accumulation if $AlCl_3$ acted on both G_p and PiPLC. However, the effects of $AlCl_3$ on CARB- and Ca^{2+}- (ionomycin) stimulated IP accumulation in synaptosomes were similar. Additionally, our data suggest that G-proteins coupled to cAMP are not affected under conditions in which IP accumulation is inhibited by $AlCl_3$ (*vide infra*).

It has been demonstrated previously that $AlCl_3$ inhibits G-protein-stimulated IP accumulation in slices [10, 11] and neuroblastoma cells [28, 29]. Aluminum ion (Al^{3+}) inhibits the activation and GTPase activity of transducin [26] and activates microtubule assembly, but inhibits GTPase activity of tubulin [27]. Aluminum fluoride (AlF^-_4) activates transducin [49], G_s [50] and G_p [51]. Finally, $AlCl_3$ enhances β-adrenergic receptor-mediated cAMP accumulation [52]. One interpretation of these results is that $AlCl_3$ alters G-protein function in a non-specific manner, and that this mechanism is responsible for its effect on IP accumulation. To investigate this possibility, we examined the inhibition of cAMP formation in cortical slices by CARB to determine if $AlCl_3$ interfered with the function of other G-proteins coupled to muscarinic receptors in our slices. CARB inhibits cAMP formation by activation of M_2 receptors that are negatively coupled to adenylate cyclase via G_i [53, 54]. Under conditions nearly identical to those in which IP accumulation is measured, there was no effect of $AlCl_3$ on forskolin-stimulated cAMP accumulation, demonstrating that $AlCl_3$ is without effect on adenylate cyclase activity. Additionally, there was no effect of $AlCl_3$ on CARB inhibition of forskolin-stimulated cAMP accumulation. Thus, whereas $AlCl_3$ affects CARB-stimulated IP accumulation coupled through G_p, it does not alter CARB-induced inhibition of cAMP accumulation coupled through G_i. These results suggest that $AlCl_3$ must have a selective effect on G_p over other G-proteins if the effect of $AlCl_3$ on IP accumulation is mediated by disruption of the G-protein function.

Activation of β-adrenergic receptors stimulates adenylate cyclase through activation of G_s. Johnson *et al.* [52] have reported that 100 μM $AlCl_3$ potentiated cAMP accumulation in cortical slices stimulated by the β-adrenergic receptor agonists isoproterenol and 2-chloroadenosine. We did not observe any effect of 500 μM $AlCl_3$ on NE-stimulated cAMP accumulation in our studies. Thus, the function of G_s is also not affected by $AlCl_3$ under the conditions of our study. Two factors may account for the disparity of the results in these two studies. Most important may be the use of freshly prepared aluminum solutions in our studies compared with the use of "aged" aluminum stock solutions by Johnson *et al.* [52]. These investigators reported that freshly prepared aluminum solutions do not have any effect on cAMP formation, whereas aluminum solutions older than 7 days increase agonist-stimulated cAMP formation [52]. Alternatively, we used phosphate-containing solutions in our experiments. Perhaps interaction with phosphate ions is sufficient for preventing interaction of aluminum with components of the cAMP system.

As reported by other investigators [32, 36, 37], we observed that changes in $[Ca^{2+}]_e$ modulated CARB-stimulated IP accumulation. Receptor-stimulated IP_3 formation results in rapid release of Ca^{2+} from intracellular stores or "calcisomes," followed by more prolonged entry of extracellular Ca^{2+}, which may serve to replenish Ca^{2+} stores and facilitate continued Ca^{2+} release (for review, see Ref. 55). It has been suggested that intracellular Ca^{2+} levels must remain elevated to prevent desensitization of receptor-mediated PiPLC activation [36]. Ca^{2+} channel antagonists, including Cd^{2+}, La^{3+}, ω-conotoxin and verapamil, decrease agonist-stimulated IP accumulation [36, 37]. $AlCl_3$ effectively competes with Ca^{2+} for binding sites on artificial membranes [13] and blocks depolarization-dependent $^{45}Ca^{2+}$ influx into synaptosomes [14]. If $AlCl_3$ competes with the availability of Ca^{2+} which modulates IP accumulation, then decreasing $[Ca^{2+}]_e$ would exacerbate the inhibitory effect of $AlCl_3$ on IP accumulation. Decreasing $[Ca^{2+}]_e$ from 1.0 to 0.1 mM, however, did not affect $AlCl_3$ inhibition of CARB-stimulated IP accumulation, suggesting that $AlCl_3$ does not interfere directly with modulation of IP accumulation by extracellular Ca^{2+}.

$AlCl_3$ has been reported to increase phos-

phorylation of cytoskeletal proteins [43] and increase partitioning of PKC to the membrane [8], suggesting that $AlCl_3$ may activate PKC. Activation of PKC by phorbol esters decreases CARB-stimulated IP accumulation [35, 41, 42], presumably by decreasing PiPLC activity or G-protein–PiPLC interactions [38, 40]. Thus, activation of PKC by $AlCl_3$ could decrease IP accumulation following receptor and G-protein activation. Our results indicate that $AlCl_3$ inhibited PKC activity in both cytosolic and membrane fractions of cortical homogenates in a concentration-dependent manner. These results are consistent with previously reported effects of aluminum on PKC activity in mouse forebrain homogenates [12]. Therefore, these data do not support the hypothesis that $AlCl_3$ inhibits CARB-stimulated IP accumulation by activating PKC. $AlCl_3$ has also been reported to inhibit Ca^{2+} extrusion by Ca^{2+}/Mg^{2+}-ATPase in synaptosomes and microsomes [15, 16]. Activation of PKC may therefore occur as a secondary effect of $AlCl_3$-induced intracellular Ca^{2+} increases in slices, resulting in decreased agonist-stimulated IP accumulation. If the decrease in CARB-stimulated IP accumulation were the result of PKC activation, then H-7, a PKC inhibitor, would alleviate the effect of $AlCl_3$, whereas PdBu, a PKC activator, would have additive effects with $AlCl_3$. In the absence of $AlCl_3$, H-7 slightly increased CARB-stimulated IP accumulation, suggesting that there is little endogenous feedback inhibition by PKC, whereas PdBu significantly decreased CARB-stimulated IP accumulation. This is consistent with previous effects of phorbol esters on agonist-stimulated IP accumulation [35, 41, 42]. However, no significant interactions were observed between $AlCl_3$ and these compounds. Thus, despite its direct effect on PKC activity and Ca^{2+}-extruding systems, direct or indirect interactions of $AlCl_3$ with PKC did not influence its inhibition of IP accumulation in cortical slices.

We have examined components of the phosphoinositide system for their susceptibility to effects of $AlCl_3$ in order to characterize mechanisms underlying acute effects of $AlCl_3$ on inositol phosphate signalling in brain slices. The ability of $AlCl_3$ to inhibit PiPLC activity in homogenates and Ca^{2+}-stimulated IP accumulation in synaptosomes suggests that inhibition of PiPLC activity is at least partially responsible for aluminum inhibition of agonist-stimulated IP accumulation. $AlCl_3$ may have a direct effect on PiPLC itself, or it may interfere with the availability of PIP_2 for hydrolysis by binding to the phosphate groups of PIP_2, as suggested by McDonald and Mamrack [30]. Alternatively, $AlCl_3$ may interfere with modulation of PiPLC activity by Ca^{2+}. Future studies will address these potential mechanisms of $AlCl_3$ inhibition of PiPLC activity.

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