Involvement of a Phorbol Ester-Insensitive Protein Kinase C in the \( \alpha_2 \)-Adrenergic Inhibition of Voltage-Gated Calcium Current in Chick Sympathetic Neurons

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\( \alpha_2 \)-Adrenergic receptors regulate the efficacy at the sympathetic-effector junction by means of a feedback inhibition of transmitter release. In chick sympathetic neurons, the mechanism involves an inhibition of N-type calcium channels, and we now present evidence that this effect involves an atypical, phorbol ester-insensitive protein kinase C (PKC). The inhibition of voltage-gated \( \text{Ca}^{2+} \) currents by the specific \( \alpha_2 \)-adrenergic agonist UK 14,304 was significantly attenuated when the PKC inhibitors PKCl(19–36), staurosporine, or calphostin C were included in the internal solution used to fill the patch pipettes, or if staurosporine or calphostin C were applied extracellularly; however, phorbol esters as classical activators of PKC or oleoylacylglycerol did not mimic the effect of UK 14,304, and chronic exposure to 4\( \beta \)-phorbol dibutyrate (PDBu) did not attenuate it, even though PKC\( \alpha \) and -z isozymes were translocated to plasma membranes by PDBu. The atypical isozyme PKC\( \zeta \) was translocated by 100 \( \mu \)M arachidonic acid (AA), but not by PDBu; 100 \( \mu \)M AA and linoleic acid inhibited voltage-activated \( \text{Ca}^{2+} \) currents, and this effect was attenuated when PKCl(19–36) was added to the patch pipette solution. Our observations indicate that classical, new, and atypical PKC isozymes are present in chick sympathetic neurons and that an atypical, phorbol ester-insensitive PKC is involved in the inhibition of voltage-activated calcium currents by \( \alpha_2 \)-adrenergic receptor activation.

Key words: \( \alpha_2 \)-adreceptor; calcium current; chick sympathetic ganglion; protein kinase C isozymes; phorbol ester; arachidonic acid

Norepinephrine reversibly inhibits voltage-gated calcium channel currents in sympathetic neurons (Horn and McAfee, 1980; Galvan and Adams, 1982; Bley and Tsien, 1990; Plummer et al., 1991; Elmslie et al., 1992) by an activation of \( \alpha_2 \)-adrenergic receptors (Schofield, 1990; Boehm and Huck, 1991; Song et al., 1991). This phenomenon has attracted considerable interest not only because it served as a paradigm for second messenger-dependent signal transduction (Beech et al., 1992; Mathie et al., 1992; Delcour and Tsien, 1993; Golard and Siegelbaum, 1993; Shapiro et al., 1994; Ehrlich and Elmslie, 1995; Ikeda, 1996), but also because a modulation of transmembrane calcium influx subsequently affects the concentration of free intracellular calcium, which is crucial for various biological processes such as transmitter release (Lipscombe et al., 1989; Miller, 1990).

Nevertheless, the signaling cascade that links \( \alpha_2 \)-adrenergic receptors and calcium channel inhibition in chick sympathetic neurons has yet to be elucidated. The effects of \( \alpha_2 \)-adrenergic receptor activation on calcium currents and on transmitter release are abolished by a pretreatment of cultures for 24 hr with pertussis toxin (Boehm et al., 1992), indicating that inhibitory \( \text{G}_{\alpha}/\text{G}_{\beta} \) G-proteins are involved. We do not know, however, whether an \( \alpha_2 \)-adrenergic receptor) activated G-protein interacts directly with calcium channels (Hescheler and Schultz, 1993) or whether second messengers are involved. In line with experiments in rat sympathetic cells (Schwartz and Malik, 1993), we have reported recently that cAMP modulates but does not mediate the inhibition of calcium channels and transmitter release by \( \alpha_2 \)-adrenergic receptor activation in chick sympathetic neurons (Boehm et al., 1994). Our experiments also indicate an intricate interaction of \( \text{G}_{\alpha} \) and the \( \alpha_2 \)-adrenergic receptor-mediated inhibition of transmitter release, because downregulation of \( \text{G}_{\alpha} \) by cholera toxin caused sensitization of \( \alpha_2 \)-adrenergic effects in chick sympathetic neurons (Boehm et al., 1996). Another obvious pathway that might convey the \( \alpha_2 \)-adrenergic effect includes activation of protein kinase C (PKC), possibly secondary to an \( \alpha_2 \)-adrenergic receptor-mediated regulation of phospholipase C (PLC) or phospholipase A\_2 (PLA\_2). Numerous reports have implicated PKC in neurotransmitter-induced modulation of \( \text{Ca}^{2+} \) channels, because both phorbol esters and diacylglycerol analogs mimicked receptor-mediated effects, and PKC inhibitors or chronic exposure to phorbol esters attenuated the action of neurotransmitters (Rane and Dunlap, 1986; Werz and Macdonald, 1987; Ewald et al., 1988; Marchetti and Brown, 1988; Mochida and Kobayashi, 1988; Rane et al., 1989; Boland et al., 1991; Diverse-Pierluissi and Dunlap, 1993).

The present experiments were initiated to investigate further the signaling cascade of the \( \alpha_2 \)-adrenergic receptor-mediated inhibition of transmitter release in cultured chick sympathetic neurons. We focused our attention on the effects of \( \alpha_2 \)-adrenergic receptor agonists on voltage-gated calcium currents and on the modulation of these effects by various substances known to activate or block the PKC system, because our previous experiments indicated that the N-type calcium channel represents the final effector of the \( \alpha_2 \)-
adrenoceptor-mediated autoinhibition of transmitter release in these neurons (Boehm and Huck, in press).

MATERIALS AND METHODS

Cell cultures. The procedures for dissociation and culture of chick sympathetic neurons have been described previously (Boehm et al., 1991, 1994). Briefly, paravertebral sympathetic ganglia were dissected from 12- to 13-day-old chick embryos, trypsinized (0.1% for 30 min at 36°C), triturated, resuspended in DMEM (Gibco 041-01885M; Life Technologies, Gaithersburg, MD) containing 2.2 g/l glucose, 10 mg/l insulin, 25,000 IU/l penicillin, and 25 mg/l streptomycin (Gibco 043-05140D), 10 μg/ml nerve growth factor (Gibco 043605B), and 5% fetal calf serum (Gibco 011-0620H), and plated on poly-D-lysine-coated (Sigma 1149; Sigma, St. Louis, MO) tissue culture dishes (Nune 153066; Nune, Naperville, IL) (~5 × 10^6 neurons per dish). Non-neural cells were reduced to <5% by differential plating, as described elsewhere (Boehm et al., 1994), when the cellular distribution of PKC isoforms was investigated.

Electrophysiology. Whole-cell Ca^{2+} currents were recorded at room temperature (20–24°C) from cell bodies of sympathetic neurons after 24–48 hr in vitro, as described elsewhere (Boehm and Huck, 1991). The internal (pipette) solution contained (in mM): 115 N-methyl-D-glucamine, 20 tetraethylammonium chloride, 1.6 CaCl_2, 2 Mg-ATP, 2 Li-GTP, 10 EGTA, 10 glucose, and 20 HEPES, adjusted to pH 7.3 with HCl, which results in a nominal calcium concentration of 0.01 μM. For applications of arachidonic acid (AA) or linoleic acid (LinA), cells were dialyzed with 200 U/ml superoxide dismutase (SOD) via the recording pipette to prevent the formation of free radicals (Chan et al., 1988; Keyser and Alger, 1990). The external (bathing) solution consisted of (in mM): 120 choline chloride, 5 CaCl_2, 20 glucose, and 10 HEPES, adjusted to pH 7.3 with KOH. Ca^{2+} currents were elicited by depolarizations from a holding potential of −80 to 0 mV at a frequency of 2–4 min^-1. To account for the time-dependent rundown of Ca^{2+} currents (see Fig. 3), drug effects were evaluated by measuring currents in the presence of test drugs (B) and by comparing them with control currents recorded before (A) and after (washout, C) the application of the drugs (Boehm and Huck, 1991), according to 200 × B/(A+C) = % of control current.

Characterization of differential distribution of PKC isoforms. Pure (>95%) neuronal cell cultures were subjected to long-term (24 hr) or short-term (10 min) treatment of a number of known PKC activators, including 4-b-phorbol dibutyrate (PDBu), oleoylacteylglycerol (OAG), AA, and LinA. As a control for PKC-independent effects of phorbol esters, the inactive isomer 4-a-phorbol dibutyrate (aPDBu) was used. Thereafter, cultures were rinsed twice with the bathing solution described above, incubated with 50 mM β-glycerophosphate, 6 mM EGTA, 5 μM leupeptin, and 1 mM phenylmethylsulfonylfluoride in bathing solution, and rapidly frozen with adding liquid nitrogen. After they were thawed, cells were scraped off the dishes and subjected to a second freeze-thaw cycle. Cytosolic and membrane fractions were separated by centrifugation (50,000 × g for 30 min at 2°C). Cytosolic proteins were precipitated with trichloroacetic acid (14% final concentration) and subsequently dissolved in Laemmli sample buffer supplemented with 2% SDS and 40 mM dithiothreitol, whereas pellets were dissolved directly in supplemented Laemmli sample buffer. Samples corresponding to 1–2.5 × 10^6 cells were applied to SDS-polyacrylamide gels (running gel: 8% acrylamide, 0.21% N,N-methylene bisacrylamide). Proteins were subsequently transferred to nitrocellulose and stained with Ponceau S to verify that comparable amounts had been loaded. The nitrocellulose blots were probed with commercially available peptide antisera specific for PKCα (Santa Cruz Biotechnology, Tebu, France, 1:200 dilution), PKCβ (Santa Cruz, 1:200 dilution), PKCγ (Life Technologies, 1:500 dilution, or Santa Cruz, 1:200). Additional isoforms were probed with antisera specific for PKCβ-II, δ, η, and θ (Santa Cruz, 1:200). The specificity of antigen–antibody reactions was checked by probing nitrocellulose blots with antibodies that had been preincubated with their corresponding immunogenic peptides. Immunostaining was carried out with a second antibody conjugated to horseradish-peroxidase using Amersham ECL-reagents (Amersham, Madison, WI). The immunoreactive bands from chick sympathetic cultures comigrated with immunoreactive bands detected in rat cerebral cortical homogenates.

Statistics. Data are given as arithmetic mean ± SEM, unless indicated otherwise; n = number of individual cells in whole-cell recordings. Significance of differences between single data points was evaluated by the unpaired Student’s t test, unless indicated otherwise.

Drugs and reagents. AA, LinA, OAG, and SOD were from Sigma (Vienna, Austria); N-(n-heptyl)-5-chloro-1-naphthalenesulfonamide (SC-10). 5-bromo-N-(4,5-dihydro-1-H-imidazol-2-yl)-6-quinolinalmine (UK 14,304) and calphostin C were from Research Bio-chemicals (Natick, MA); aPDBu and PDBu, the pseudosubstrate peptide inhibitor of PKC [PKCI(19–36)], a noninhibitory analog peptide [glu27, PKCI(19–36)], and a peptide inhibitor of PKA [PKI(6–22)amide] were from Life Technologies.

RESULTS

Effects of protein kinase inhibitors on the α2-adrenergic inhibition of Ca^{2+} currents

In line with our previous observations (Boehm and Huck, 1991), the specific α2-adrenoceptor agonist UK 14,304 reversibly inhibited voltage-activated calcium currents (Figs. 1, 2). The effect of UK 14,304 was reduced progressively within minutes when the PKC pseudosubstrate inhibitor PKCI(19–36) (10 μM) (House and Kemp, 1987) was included in the pipette solution (Figs. 1 A, B). Peak calcium current amplitudes in the absence of UK 14,304 were not affected significantly by PKCI(19–36) [control: 756 ± 90 pA, n = 18; PKCI(19–36): 633 ± 69 pA, n = 17; p > 0.25]. Likewise, the dialysis of neurons with calphostin C and staurosporine (both at 1 μM) for 10 min significantly diminished the α2-adrenoceptor inhibition (Fig. 2A, B). A noninhibitory analog peptide [glu27,PKCI(19–36)] and a selective peptide inhibitor of PKA [PKAI(6–22)amide] (Glass et al., 1989) had no effect on the UK 14,304-induced inhibition (Figs. 1 A, B, 2B). Even under conditions in which these substances effectively blocked the action of UK 14,304, neither PKCI(19–36) nor calphostin C or staurosporine had any noticeable effect on the somatostatin-induced inhibition of calcium currents (Figs. 1, 2 A). In chick sympathetic neurons, staurosporine has been shown previously to block the calcium current inhibition by somatostatin, although activation of PKC (by pretreatment with OAG or phorbol-12-myristate-13-acetate) significantly reduced the effects of somatostatin (Golard et al., 1993). Because both substances are membrane-permeable, we could apply staurosporine and calphostin C extracellularly after the effects of UK 14,304 had initially been established. Hence, in neurons exposed to 1 μM staurosporine or calphostin C, the subsequent inhibition by UK 14,304 in the continuing presence of either PKC inhibitor was reduced significantly (inhibition before staurosporine or calphostin C: 34.6 ± 2.2%, n = 8; after >3 min staurosporine: 11.6 ± 3.0%, n = 4; p < 0.001, after >3 min calphostin C: 18.8 ± 3.0%, n = 4; p < 0.05). To investigate further the role of PKC, a subset of cultures was treated with 10 μM PDBu for 24 hr. Long-lasting exposure of neurons to active phorbol esters downregulates PKC and results, for example, in a marked reduction of stimulated transmitter release (Matthies et al., 1987) or an attenuation of neuropeptide Y-mediated inhibition of voltage-activated calcium currents (Ewald et al., 1988). This treatment, however, failed to alter the α2-adrenergic inhibition of Ca^{2+} currents (Fig. 2B).

Effects of PKC activators on Ca^{2+} currents and on the α2-adrenergic inhibition

Our observation that the effects of UK 14,304 on voltage-activated calcium currents were attenuated by inhibitors of PKC, but were independent of a phorbol ester-induced PKC downregulation, indicate the involvement of phorbol ester-insensitive PKC isoforms. In line with this hypothesis, we found no effect of extra-
cellularly applied 3 μM PDBu, 30 μM OAG, or 10 μM SC-10 (an activator of Ca2+-dependent PKC isoforms) (Ito et al., 1986) on voltage-gated calcium currents (Fig. 3). In addition, neither intracellular (Fig. 2) nor extracellular (not shown) applications of 1 μM PDBu significantly reduced the α2-adrenergic inhibition.

By contrast, the application of 100 μM AA (Fig. 3A,B) or LinA (Fig. 3B) for 1 min reduced Ca2+ currents in a partially reversible manner. Both AA and LinA have been shown previously to activate not only classical but also atypical, phorbol ester-insensitive PKCs (Asaoka et al., 1992; Nishizuka, 1992; Nakanishi and Exton, 1992). Lower concentrations of AA (30 μM) were also effective (not shown) but less well distinguishable from the rundown of current that occurs in this and other preparations to a variable extent (Kostyuk et al., 1981). The effects of AA and LinA were reduced significantly when 10 μM PKCI was added to the patch pipette solution (Fig. 3A,B).

Figure 1. The PKC inhibitor PKCI(19–36) prevents the α2-adrenoceptor-mediated but not the somatostatin-mediated inhibition of Ca2+ currents. A. Calcium currents were induced from a holding potential of −80 mV by depolarizing pulses to 0 mV. UK (10 μM) or somatostatin (1 μM) were applied to the same cells 3, 7, and 12 min after establishing the whole-cell condition. The recording pipette contained 10 μM peptide inhibitor against protein kinase C [PKCI(19–36)] (left panel) or 10 μM peptide inhibitor against protein kinase A [PKI(6–22)amide] (right panel). Traces show currents before, during, and after application of agonists. Calibration: 0.5 nA, 30 msec. B. Time-plots of recordings from the cells shown above in A. Filled symbols indicate test pulses in the absence or presence of 10 μM UK 14,304 (U) or 1 μM somatostatin (S).
At least 12 distinct subtypes of PKC have been characterized and categorized further into three groups (Nishizuka, 1992; Dekker and Parker, 1994): groups A and B comprise “classical” and “new” enzymes, all of which can be activated by active phorbol esters or diacylglycerol analogs. By contrast, group C consists of “atypical” PKC enzymes, which are insensitive to diacylglycerol and phorbol esters (Nishizuka, 1992). In view of the inhibitory action of PKC inhibitors and the lack of effects of phorbol esters on the α2-adrenoceptor-mediated inhibition of calcium currents, we investigated which of the various isoforms of PKC were present in chick sympathetic neurons. Immunoblot analysis with peptide antisera specific for PKC isoforms revealed the presence of α, βII, δ, ε, and ζ, but not η and θ, in both the cytosolic and membrane fractions obtained from pure neuronal cell cultures (not shown). Subsequently, PKCα, ε, and ζ as representatives of classical, new, and atypical PKC subtypes, respectively (Nishizuka, 1992), were investigated further.

PKCα and ε migrated with an apparent molecular mass of ~80 and ~90–95 kDa, respectively. Immunoblots with antisera specific for PKCζ from two different sources (Life Technologies and Santa Cruz) detected two bands migrating with an estimated molecular mass of ~68 kDa; the enzyme purified from renal tissue migrated at ~78 kDa (Nakanishi and Exton, 1992), and expression of the cDNA directed the synthesis of a ~64 kDa protein in COS-7 cells (Ono et al., 1989) and of an ~80 kDa protein in insect and mammalian cells (Liyanage et al., 1992). Thus, it is not clear whether the 72 kDa band observed in sympathetic neurons corresponds to a post-translationally modified PKCζ (e.g., by phosphorylation and/or proteolytic cleavage, known to occur in vivo) (Hug and

### Figure 2. α2-Adrenoceptor-mediated inhibition of Ca2+ currents: summary of effects of PKC inhibitors. A, Inhibition of Ca2+ currents by 10 μM UK 14,304 (left) or 1 μM somatostatin (right) in cells dialyzed for at least 10 min with 0.1% DMSO, 1 μM calphostin C, or 1 μM staurosporine. Traces show currents before, during, and after the application of agonists. Calibration: 0.5 nA for DMSO, 0.25 nA for staurosporine, 0.1 nA for calphostin C; 30 msec. B, Inhibition of peak Ca2+ current amplitudes (ICₘ) by 10 μM UK 14,304 under control conditions (cI) or with 10 μM PKCl(19–36), a peptide inhibitor of protein kinase C (PKC); 10 μM glu₂⁷-PKCl(19–36), a noninhibitory analog peptide (PKCnI); 10 μM PKAl(6–22)amide, a peptide inhibitor of PKA (PKAI); 0.1% DMSO (DMSO); 1 μM calphostin C (calph); 1 μM staurosporine (stau); or 1 μM PDBu added to the pipette solution. Alternatively, cells pretreated for 24 hr with 10 μM β-phorbol-12,13-dibutyrate (24 hr PDBu) were tested with 10 μM UK 14,304 using regular pipette solution. Currents were recorded 10 min or later after breaking the cell membrane. **, p < 0.01 vs control; ##, p < 0.01 vs DMSO; n indicated in the bars.
Sarre, 1993), or to a different atypical PKC-isofrom. PKC, for instance, shows not only a 72% identity with PKCz, but an antibody raised against PKCz also recognizes PKCi (Selbie et al., 1993). For practical purposes, we subsequently refer to both bands as PKCz.

A 10 min incubation of neurons with 1 μM of the active phorbol ester PDBu induced an almost complete translocation of PKCa and PKCe from the cytoplasm to the membrane, whereas the distribution of the PKCz-bands was unaffected (Fig. 4). Exposure of the neurons to 30 μM OAG, 3 μM PDBu, 10 μM SC-10, 100 μM AA, or 100 μM LinA, calculated as % inhibition = 100 – (200 b/a + c), where a, b, and c are the current amplitudes measured after 60, 120, and 180 sec, respectively, as indicated in A. Neurons were dialyzed for at least 10 min with standard pipette solution (open bars), with SOD (hatched bars), or with SOD plus 10 μM of the peptide inhibitor PKCII(19–36) (filled bars), which significantly attenuates the effects of AA and LinA. Levels of significance for the difference between corresponding bars are indicated, n = 5–6.

DISCUSSION

Inhibitors of PKC antagonize the effect of UK 14,304, but phorbol esters have no effect on calcium current and on the α2-adrenoceptor-mediated modulation

In line with our previous observations, the activation of α2-adrenoceptors by UK 14,304 caused an inhibition of voltage-activated calcium current in chick sympathetic neurons (Boehm and Huck, 1991). This inhibition was reduced significantly when the pseudosubstrate peptide inhibitor of PKC [PKCII(19–36), 10 μM] (House and Kemp, 1987) was included in the pipette solution. A noninhibitory analogous peptide [glu27.PKCII(19–36)] had no effect. Staurosporine and calphostin C, two membrane-permeant inhibitors of PKC, exerted effects similar to those of PKCII(19–
regardless of whether they were applied through the recording pipette or extracellularly.

These results imply that PKC is part of the signaling cascade that leads to an inhibition of voltage-gated calcium channels by

![Figure 4](image)

**Figure 4.** Translocation of PKCα, ε, and ζ isoforms by phorbolesters and AA. The distribution of protein kinase C isoforms was investigated by immunoblots after a 10 min incubation of cultures with 100 μM AA, with the inactive phorbolester αPDBU, or with the active phorbolester βPDBU, both at 1 μM. Cells were lysed as described in Materials and Methods; aliquots of the pellet and supernatant corresponding to 2 × 10⁵ cells were applied to SDS-polyacrylamide gels. Immunoblots were probed with peptide antisera specific for PKCα (A), PKCe (B), and PKCζ (C). Standard: 10 μg protein from rat neocortical homogenate. Arrows indicate the position of bands that were suppressed when antibodies had been preincubated with the corresponding immunogenic peptide (not shown). Data are representative of three additional experiments performed on different preparations.

![Figure 5](image)

**Figure 5.** Downregulation of classical, but not atypical, PKC isoforms by phorbolesters. The distribution of protein kinase C isoforms was analyzed by immunoblots after a 24 hr treatment of cultures with 100 μM AA, with 1 μM of the inactive and active phorbolesters αPDBU and βPDBU, respectively, or with the vehicle 0.1% DMSO. Cells were lysed as described in Materials and Methods; aliquots of the pellet and supernatant corresponding to 2 × 10⁵ cells were applied to SDS-polyacrylamide gels. Immunoblots were probed with peptide antisera specific for PKCα (A), PKCe (B), and PKCζ (C). Arrows indicate the position of bands that were blocked when antibodies had been preincubated with the corresponding immunogenic peptide (not shown). Data are representative of three additional experiments performed on different preparations.
UK 14,304. In fact, numerous reports have provided evidence that effects of neurotransmitter receptor activation on calcium currents involve PKC (Diverse-Pierluissi and Dunlap, 1993). Neither the phorbol ester PDBu nor the synthetic diacylglycerol analog OAG, however, were able to mimic the effects of UK 14,304, and downregulation of PKC by a 24 hr incubation of cultures with PDBu did not attenuate the inhibition of calcium currents by UK 14,304. In rat sympathetic neurons, OAG (50 μM) was reported to inhibit calcium currents, but because L-type currents were affected along with N-type currents, and because PKC inhibitors like PKC1(19–31) did not affect transmitter-mediated inhibitions of calcium currents, a direct involvement of PKC on both transmitter- and OAG-mediated effects was ruled out (Plummer et al., 1991). Bley and Tsien (1990) also found no evidence for a PKC-mediated pathway of a peptidergic inhibition in frog sympathetic neurons, because phorbol esters did not mimic the inhibition of calcium currents and the PKC inhibitor staurosporine did not block the peptidergic effects. By contrast, the summary of our observations implies a phorbol ester-insensitive (atypical) PKC as part of the signaling cascade of α2-adrenoceptor-mediated effects in chick sympathetic neurons.

At present, we cannot exclude the possibility that additional signaling pathways independent of PKC may be involved, particularly because a small inhibition of calcium currents by UK 14,304 persisted in the presence of PKC1(19–36). In chick sensory neurons, α2-adrenoceptors couple to two types of pertussis toxin-sensitive G-proteins and use two separate pathways to regulate N-channel function. One includes G-protein βγ-subunits as well as PKC, whereas the second signal cascade seems to be independent of PKC but involves Go (Diverse-Pierluissi et al., 1995).

**Phorbol ester-sensitive and -insensitive PKC isoforms are expressed in chick sympathetic neurons**

To date, three major groups of PKC isoforms have been characterized (Asaoka et al., 1992; Nishizuka, 1992; Dekker and Parker, 1994): groups A and B comprise “classical” and “new” enzymes, all of which can be activated by active phorbol esters or diacylglycerol analogs. Group C, by contrast, consists of “atypical” PKC enzymes that are insensitive to diacylglycerol and phorbol esters (Nishizuka, 1992).

PKCα and PKCε, as representatives of classical and new PKC isoforms, responded to short-term incubations of cultures with PDBu and OAG by a translocation from the cytosol to the membrane, indicating that phorbol ester-sensitive PKC isoforms are present in the chick sympathetic neurons. Our data also indicate the presence of “atypical” PKCγ, which was translocated by AA but not by PDBu or OAG. All subtypes of PKC are likely to be inhibited by PKCI (House and Kemp, 1987) and staurosporine (Nakadate et al., 1988), both of which act at the catalytic domain highly conserved between all known PKC subtypes (Nishizuka, 1992; Hug and Sarre, 1993). Likewise, calphostin C, although acting at the regulatory domain of PKCs, seems to inhibit a broad range of PKC isoforms, because it reduced the activity of Ca2+-independent PKC isoforms at least as potently as the classical Ca2+-dependent isoforms (Ozawa et al., 1993). Hence, we may expect that PKCI, staurosporine, and calphostin C all inhibit not only classical isoforms of PKC but also atypical forms unresponsive to phorbol esters. Our experiments indicate that such isoforms are present in chick sympathetic neurons and may therefore mediate the α2-adrenergic inhibition of calcium currents in a phorbol ester-insensitive manner. PKCγ has been demonstrated in nervous tissue and has been suggested as playing a role in the maintenance of long-term potentiation in the hippocampal CA1 region (Sacktor et al., 1993).

**AA induces translocation of all PKC isoforms and inhibits voltage-activated calcium channel currents**

AA and related fatty acids are known, but not exclusive, activators of most PKC isoforms (Asaoka et al., 1992; Nishizuka, 1992). Our experiments indicate that both short- and long-term incubations of cultures with 100 μM AA induced translocation of PKCα and PKCε as well as PKCζ from the cytosol to the membrane fraction in chick sympathetic cultures. Extracellular applications of 100 μM AA or 100 μM LinA also inhibited voltage-activated calcium currents, and these effects were attenuated by >50% when PKC1(19–36) was included in the pipette solution, indicating a PKC-specific component of the phenomenon. Residual effects in the presence of the PKC inhibitor might be attributable to a straight action on calcium channels, because AA also affects transmembrane ion channels directly (Ordway et al., 1991; Fraser et al., 1993; Meves, 1994).

**The link between α2-adrenoceptors and the activation of atypical PKC remains to be identified**

Free AA, which may be generated by receptor-mediated activation of PLA2 (Axelrod, 1990), exerts a plethora of effects in the nervous system by the activation of PKC (Axelrod et al., 1988; Keyser and Alger, 1990; Meves, 1994). We therefore tested whether UK 14,304 would enhance cellular levels of AA, thus making AA not only a pharmacological tool but also a physiological candidate in the signal transduction pathway between α2-adrenoceptors and the activation of atypical PKC. Our experiments were inconclusive, however, because short-term incubations with UK 14,304 enhanced the generation of free AA in only 7 of 12 experiments (S. Boehm, S. Huck, and M. Freimuth, unpublished observations). In addition, the high concentrations required to inhibit calcium currents render AA an unlikely candidate to mediate the effect of UK 14,304. Hence, in the signaling cascade α2-adrenoceptors—activation of an atypical PKC—inhibition of calcium currents, as delineated in this description for chick sympathetic neurons, the missing link or links between α2-adrenoceptors and atypical PKC remain to be identified.

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