Involvement of a Phorbol Ester-Insensitive Protein Kinase C in the α_2-Adrenergic Inhibition of Voltage-Gated Calcium Current in Chick Sympathetic Neurons

Stefan Boehm,[1,2] **Sigismund Huck,**[1] **and Michael Freissmuth**[2]

Departments of ¹Neuropharmacology and ²Pharmacology, University of Vienna, A-1090 Vienna, Austria

α_2-Adrenoceptors regulate the efficacy at the sympatho-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 Ca^{2+} currents by the specific α_2-adrenergic agonist UK 14,304 was significantly attenuated when the PKC inhibitors PKCI(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 oleoylacetylglycerol did not mimic the effect of UK 14,304, and chronic exposure to 4-β-phorbol dibutyrate (PDBu) did not attenuate it, even though PKCα and -ϵ isozymes were translocated to plasma membranes by PDBu. The atypical isozyme PKCζ was translocated by 100 μM arachidonic acid (AA), but not by PDBu; 100 μM AA and linoleic acid inhibited voltage-activated Ca^{2+} currents, and this effect was attenuated when PKCI(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 α_2-adrenoceptor activation.

Key words: α_2-adrenoceptor; 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 at al., 1992) by an activation of α_2-adrenoceptors (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 α_2-adrenoceptors and calcium channel inhibition in chick sympathetic neurons has yet to be elucidated. The effects of α_2-adrenoceptor 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 G_o/G_i G-proteins are involved. We do not know, however, whether an (α_2-adrenoceptor) 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 α_2-adrenoceptor activation in chick sympathetic neurons (Boehm et al., 1994). Our experiments also indicate an intricate interaction of $G_{s\alpha}$ and the α_2-adrenoceptor-mediated inhibition of transmitter release, because downregulation of $G_{s\alpha}$ by cholera toxin caused sensitization of α_2-adrenergic effects in chick sympathetic neurons (Boehm et al., 1996).

Another obvious pathway that might convey the α_2-adrenergic effect includes activation of protein kinase C (PKC), possibly secondary to an α_2-adrenoceptor-mediated regulation of phospholipase C (PLC) or phospholipase A_2 (PLA$_2$). Numerous reports have implicated PKC in neurotransmitter-induced modulation of 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 α_2-adrenoceptor-mediated inhibition of transmitter release in cultured chick sympathetic neurons. We focused our attention on the effects of α_2-adrenoceptor 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 α_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-d-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/l nerve growth factor (Gibco 0436050), and 5% fetal calf serum (Gibco 011-0620H), and plated on poly-D-lysine-coated (Sigma 1149; Sigma, St. Louis, MO) tissue culture dishes (Nunc 153066; Nunc, Naperville, IL) (~5 × 10⁵ 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 0.011 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.

Determination of the cellular 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-β-phorbol dibutyrate (PDBu), oleoylacetylglycerol (OAG), AA, and LinA. As a control for PKC-independent effects of phorbol esters, the inactive isomer 4-α-phorbol dibutyrate (αPDBu) 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 by 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 × 10⁵ 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), PKCε (Santa Cruz, 1:200 dilution), PKCζ (Life Technologies, 1:500 dilution, or Santa Cruz, 1:200). Additional isoforms were probed with antisera specific for PKC βI/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-quinoxalinamine (UK 14,304), staurosporine, and calphostin C were from Research Biochemicals (Natick, MA); αPDBu 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.

SC-10 (10 mM), staurosporine (1 mM), calphostin C (1 mM), and OAG (30 mM) were dissolved initially in dimethyl sulfoxide (DMSO); AA (100 mM) and LinA (100 mM) were dissolved in ethanol. Drugs were then diluted to the final concentrations (1:1000) in buffer, and appropriate controls verified that neither DMSO nor ethanol at 0.1% affected the parameters tested.

RESULTS

Effects of protein kinase inhibitors on the $α_2$-adrenergic inhibition of Ca^{2+} currents

In line with our previous observations (Boehm and Huck, 1991), the specific $α_2$-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*, 2*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 $α_2$-adrenergic inhibition (Fig. 2*A,B*). A noninhibitory analogous 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*, 2*B*). 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 not 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* < 0.01).

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 $α_2$-adrenergic inhibition of Ca^{2+} currents (Fig. 2*B*).

Effects of PKC activators on Ca^{2+} currents and on the $α_2$-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-
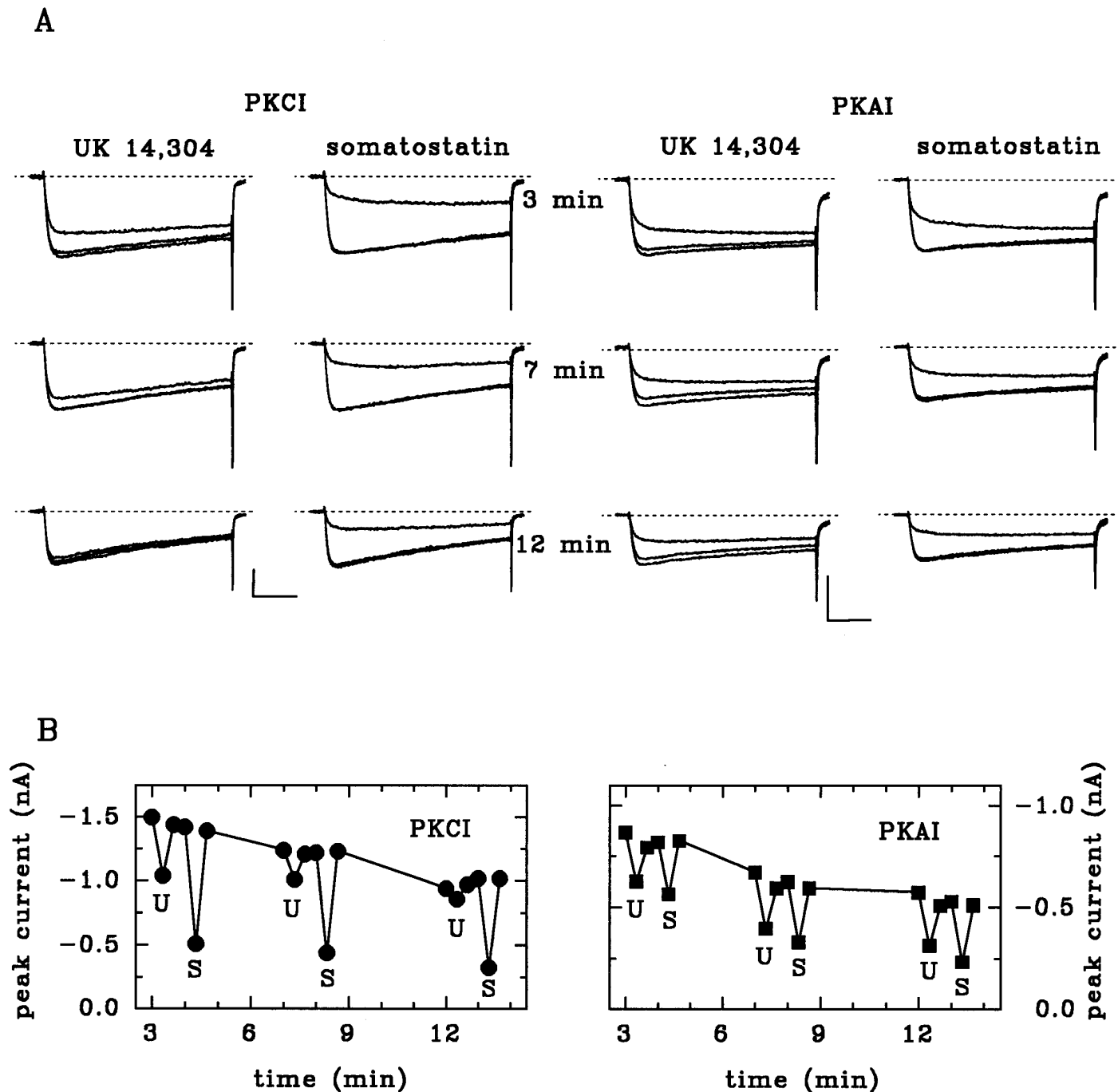
Figure 1. The PKC inhibitor PKCI(19–36) prevents the α_2-adrenoceptor-mediated but not the somatostatin-mediated inhibition of Ca^{2+} currents. *A*, Calcium currents were induced from a holding potential of -80 mV by depolarizing pulses to 0 mV. UK (10 μM) or somatostatin (1 μM) were applied to the 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*).

cellularly applied 3 μM PDBu, 30 μM OAG, or 10 μM SC-10 (an activator of Ca^{2+}-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 α_2-adrenergic inhibition.

By contrast, the application of 100 μM AA (Fig. 3*A,B*) or LinA (Fig. 3*B*) for 1 min reduced Ca^{2+} 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. 3*A,B*).
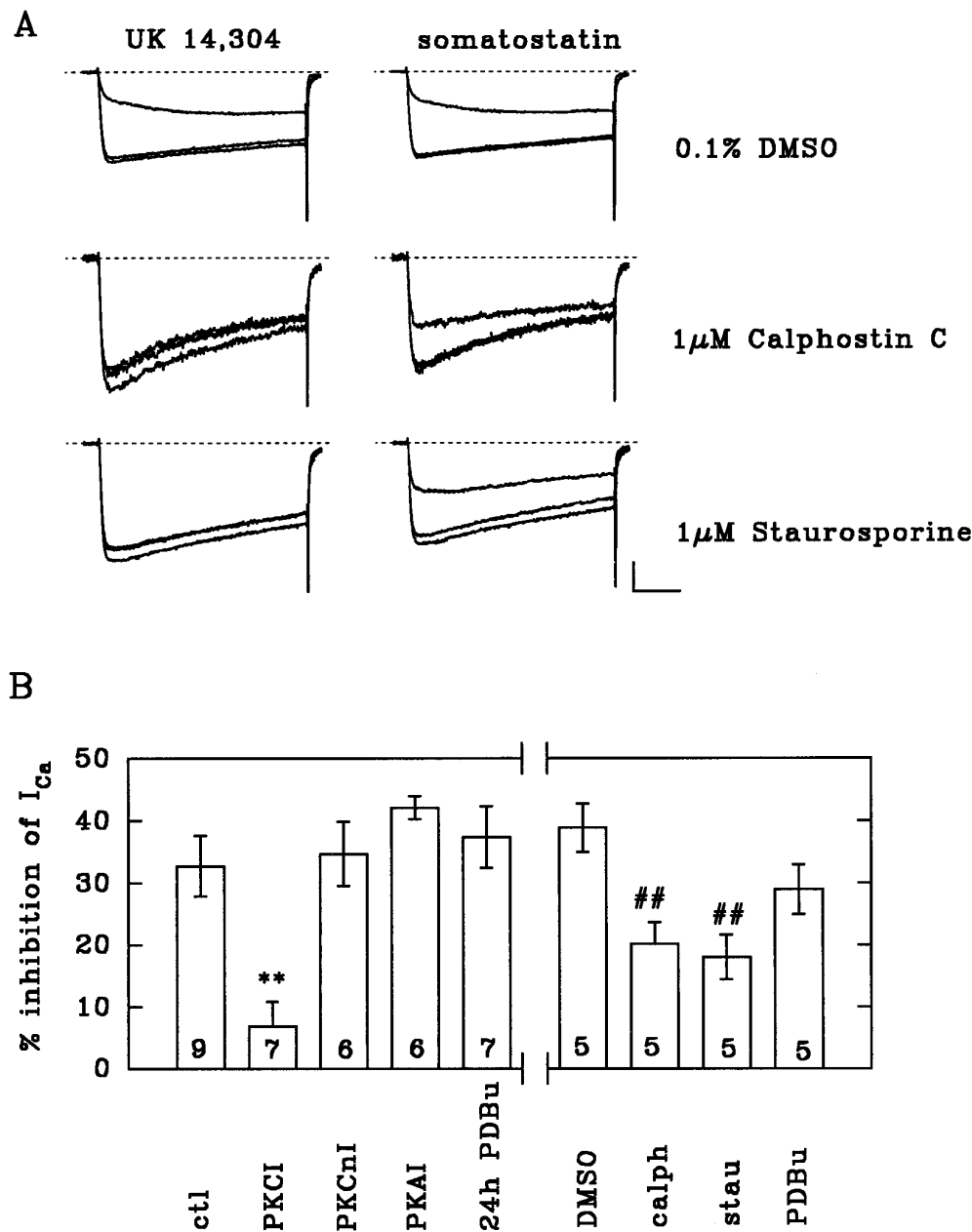
Figure 2. α_2-Adrenoceptor-mediated inhibition of Ca^{2+} currents: summary of effects of PKC inhibitors. *A*, Inhibition of Ca^{2+} currents by 10 μM UK 14,304 (*left*) or 1 μM somatostatin (*right*) in cells dialyzed for at least 10 min with 0.1% DMSO, 1 μM calphostin C, or 1 μM staurosporine. *Traces* 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 Ca^{2+} current amplitudes (I_{Ca}) by 10 μM UK 14,304 under control conditions (*ctl*) or with 10 μM PKCI(19–36), a peptide inhibitor of protein kinase C (*PKCI*); 10 μM glu[27]-PKCI(19–36), a noninhibitory analog peptide (*PKCnI*); 10 μM PKAI(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*.

Pharmacological sensitivities of PKC isoforms in chick sympathetic neurons

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 α_2-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, βI/II, 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 $\sim$80 and $\sim$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 72 and 78 kDa (Figs. 4, 5). These bands were also detected in homogenates (Fig. 4) and in membrane fractions from rat neocortex (not shown). The predicted molecular mass of PKCζ is 68 kDa; the enzyme purified from renal tissue migrated at $\sim$78 kDa (Nakanishi and Exton, 1992), and expression of the cDNA directed the synthesis of a $\sim$64 kDa protein in COS-7 cells (Ono et al., 1989) and of an $\sim$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
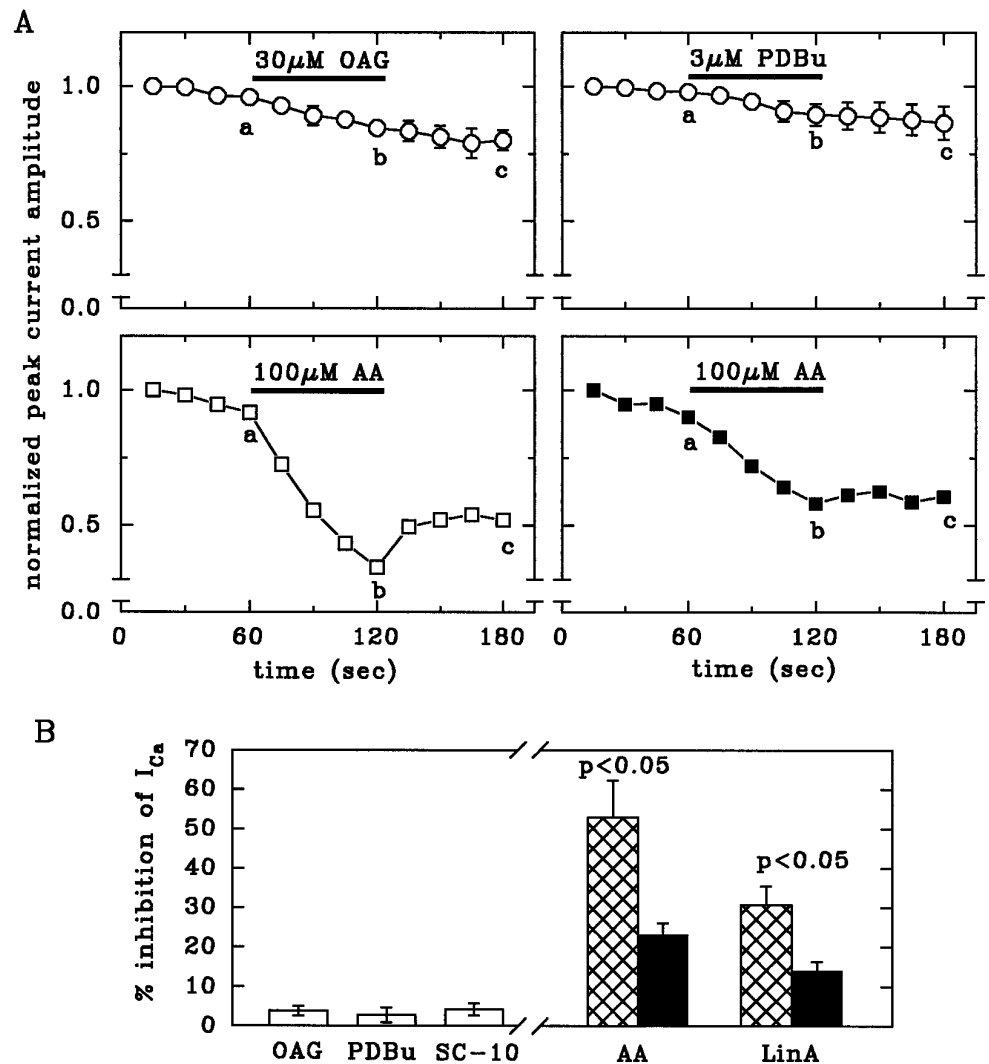
Figure 3. AA and LinA, but not phorbol esters or OAG, inhibit Ca^{2+} currents. *A*, Time course of peak current amplitudes (normalized to the first amplitude) and effects of OAG, PDBu, and AA. These drugs were applied to cells dialyzed for at least 10 min with standard pipette solution (*open circles*), with 200 U/ml SOD (*open squares*), or with SOD plus 10 μM of the peptide inhibitor PKCI(19–36) (*filled squares*). $n = 5–6$. *B*, Inhibition of Ca^{2+} currents by 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 PKCI(19–36) (*filled bars*), which significantly attenuates the effects of AA and LinA. Levels of significance for the difference between corresponding bars are indicated. $n = 5–6$.

Sarre, 1993), or to a different atypical PKC-isoform. PKC, for instance, shows not only a 72% identity with PKCζ, but an antibody raised against PKCζ also recognizes PKCι (Selbie et al., 1993). For practical purposes, we subsequently refer to both bands as PKCζ.

A 10 min incubation of neurons with 1 μM of the active phorbol ester PDBu induced an almost complete translocation of PKCα and PKCϵ from the cytoplasm to the membrane, whereas the distribution of the PKCζ-bands was unaffected (Fig. 4). Exposure of the neurons to 30 μM OAG mimicked the effects of PDBu (not shown). αPDBu, the inactive analog of PDBu, did not affect the distribution of any of the PKC subtypes (Fig. 4). AA (100 μM), which also activates PKCζ (Nakanishi and Exton, 1992), translocated all three isoforms from the cytosol to the membrane (Fig. 4).

Long-term exposure (24 hr) of cultures to PDBu (1 μM), but not αPDBu, eliminated PKCα from both the cytoplasmic and the membrane fraction (Fig. 5*A*). The response of PKC was variable: in two of four neuronal cultures, the levels of PKCϵ were essentially unaffected (Fig. 5*B*), whereas a clear-cut decline was observed in the two remaining experiments (not shown). The reason for this variability is not clear but is indicative of a relative refractoriness of PKCϵ to phorbol ester-dependent downregulation. The extent to which PKCϵ can be downregulated by phorbol

esters is known to vary in different cell lines (Hug and Sarre, 1993). Unlike PKCα and ϵ, PKCζ-bands were affected in no instance by long-term incubation with the phorbol ester (Fig. 5*C*).

By contrast to the downregulation of PKCα and PKCϵ by PDBu, a 24 hr exposure to 100 μM AA consistently *increased* the immunoreactivity for all three PKC isoforms (α, ϵ, and ζ) in the particulate fraction. This indicates that AA induced a persistent translocation of the three different PKC subtypes to the membranes but failed to downregulate any of the enzymes (Fig. 5).

DISCUSSION

Inhibitors of PKC antagonize the effect of UK 14,304, but phorbol esters have no effect on calcium current and on the α_2-adrenoceptor-mediated modulation

In line with our previous observations, the activation of α_2-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 [PKCI(19–36), 10 μM] (House and Kemp, 1987) was included in the pipette solution. A noninhibitory analogous peptide [glu^{27}-PKCI(19–36)] had no effect. Staurosporine and calphostin C, two membrane-permeant inhibitors of PKC, exerted effects similar to those of PKCI(19–
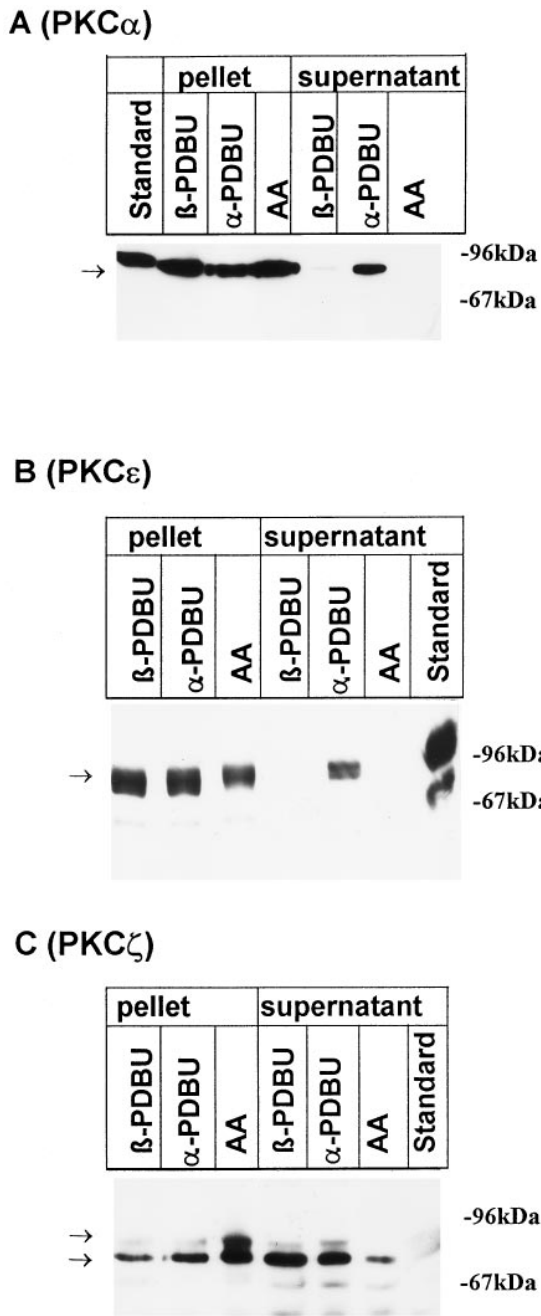
Figure 4. Translocation of PKCα, ε, and ζ isozymes by phorbol esters and AA. The distribution of protein kinase C isoforms was investigated by immunoblots after a 10 min incubation of cultures with 100 μM *AA*, with the inactive phorbol ester *αPDBU*, or with the active phorbol ester *βPDBU*, both at 1 μM. Cells were lysed as described in Materials and Methods; aliquots of the pellet and supernatant corresponding to 2×10^5 cells were applied to SDS-polyacrylamide gels. Nitrocellulose blots were probed with peptide antisera specific for PKCα (*A*), PKCε (*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.

36), 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
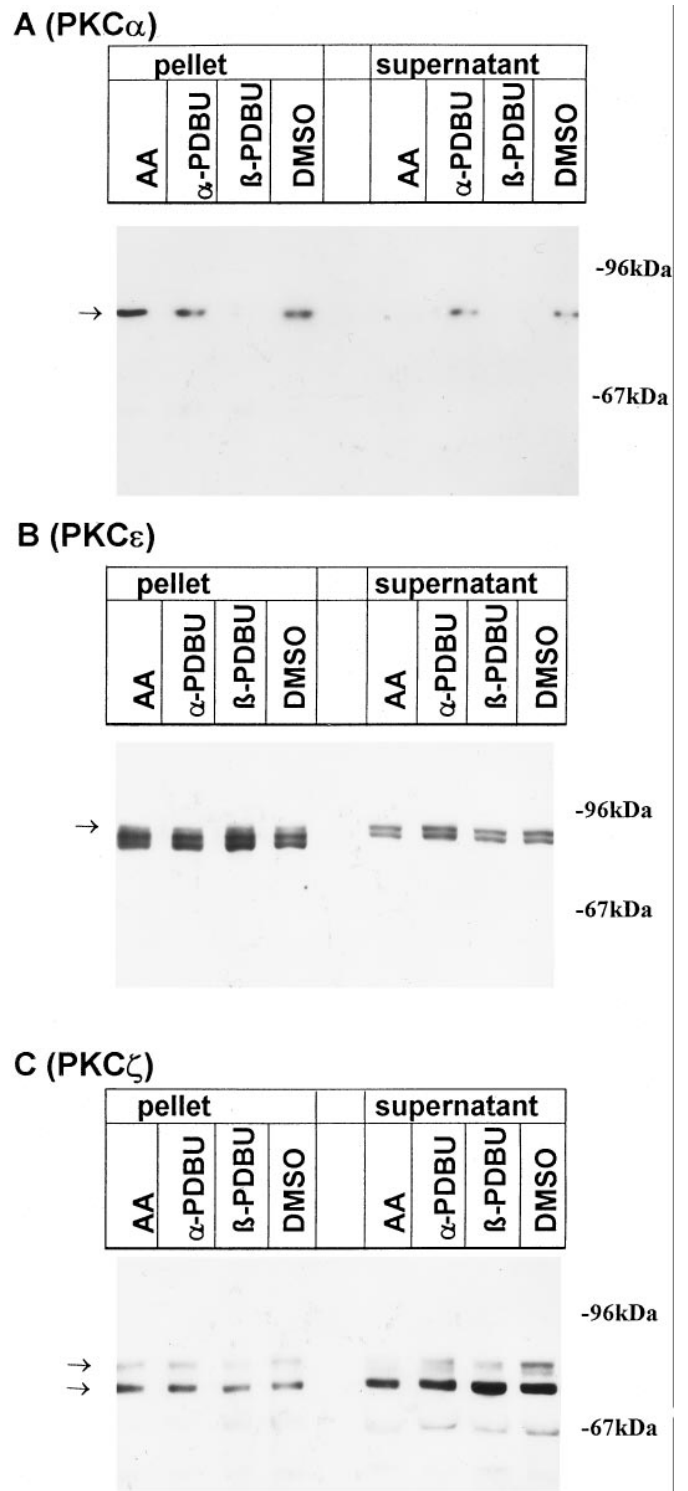

Figure 5. Downregulation of classical, but not atypical, PKC isozymes by phorbol esters. The distribution of protein kinase C isoforms was analyzed by immunoblots after a 24 hr treatment of cultures with 100 μM *AA*, with 1 μM of the inactive and active phorbol esters *αPDBU* and *βPBDU*, respectively, or with the vehicle 0.1% DMSO. Cells were lysed as described in Materials and Methods; aliquots of the pellet and supernatant corresponding to 2×10^5 cells were applied to SDS-polyacrylamide gels. Nitrocellulose blots were probed with peptide antisera specific for PKCα (*A*), PKCε (*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 PKCI(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 α_2-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 PKCI(19–36). In chick sensory neurons, α_2-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 $\beta\gamma$-subunits as well as PKC, whereas the second signal cascade seems to be independent of PKC but involves $G_{o\alpha}$ (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 isozymes 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" PKCζ, 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 Ca^{2+}-independent PKC isozymes at least as potently as the classical Ca^{2+}-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 α_2-adrenergic inhibition of calcium currents in a phorbol ester-insensitive manner. PKCζ 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 isozymes and inhibits voltage-activated calcium channel currents

AA and related fatty acids are known, but not exclusive, activators of most PKC isozymes (Asaoka et al., 1992; Nishizuka, 1992). Our experiments indicate that both short- and long-term incubations of cultures with 100 μM AA induce 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 PKCI(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 α_2-adrenoceptors and the activation of atypical PKC remains to be identified

Free AA, which may be generated by receptor-mediated activation of PLA$_2$ (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 α_2-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. Freissmuth, 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 α_2-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 α_2-adrenoceptors and atypical PKC remain to be identified.

REFERENCES

Asaoka Y, Nakamura S-I, Yoshida K, Nishizuka Y (1992) Protein kinase C, calcium and phospholipid degradation. Trends Biochem Sci 17:414–417.

Axelrod J (1990) Receptor-mediated activation of phospholipase A$_2$ and arachidonic acid release in signal transduction. Biochem Soc Trans 18:503–507.

Axelrod J, Burch RM, Jelsema CL (1988) Receptor-mediated activation of phospholipase A$_2$ via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. Trends Neurosci 11:117–123.

Beech DJ, Bernheim L, Hille B (1992) Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. Neuron 8:97–106.

Bley KR, Tsien RW (1990) Inhibition of Ca^{2+} and K^+ channels in sympathetic neurons by neuropeptides and other ganglionic transmitters. Neuron 2:379–391.

Boehm S, Huck S (1991) Modulation of calcium currents via α_2-adrenoreceptors in embryonic chick sympathetic neurons. Naunyn Schmiedebergs Arch Pharmacol 344:382–385.

Boehm S, Huck S (1996) Inhibition of N-type calcium channels: the only mechanism by which presynaptic α2-autoreceptors control sympathetic transmitter release. Eur J Neurosci, in press.

Boehm S, Huck S, Drobny H, Singer EA (1991) Electrically evoked noradrenaline release from cultured chick sympathetic neurons: mod-

ulation via presynaptic α_2-adrenoceptors and lack of autoinhibition. Naunyn Schmiedebergs Arch Pharmacol 344:130–132.

Boehm S, Huck S, Drobny H, Singer EA (1992) Pertussis toxin abolishes the inhibition of Ca^{2+} currents and of noradrenaline release via α_2-adrenoreceptors in chick sympathetic neurons. Naunyn Schmiedebergs Arch Pharmacol 345:606–609.

Boehm S, Huck S, Koth G, Agneter E, Drobny H, Singer EA (1994) α_2-Adrenoceptor-mediated inhibition of electrically evoked [^{3}H]noradrenaline release from chick sympathetic neurons: role of cyclic AMP. J Neurochem 63:146–154.

Boehm S, Huck S, Motejlek A, Drobny H, Singer EA, Freissmuth M (1996) Cholera toxin induces cyclic AMP-independent downregulation of $G_{s\alpha}$-autoreceptors in chick sympathetic neurons. J Neurochem 66:1019–1026.

Boland LM, Allen AC, Dingledine R (1991) Inhibition by bradykinin of voltage-activated barium current in a rat dorsal root ganglion cell line: role of protein kinase C. J Neurosci 11:1140–1149.

Chan PH, Chen SF, Yu ACH (1988) Induction of intracellular superoxide radical formation by arachidonic acid and by polyunsaturated fatty acids in primary astrocytic cultures. J Neurochem 50:1185–1193.

Dekker LV, Parker PJ (1994) Protein kinase C: a question of specificity. Trends Biochem Sci 19:73–77.

Delcour AH, Tsien RW (1993) Altered prevalence of gating modes in neurotransmitter inhibition of N-type calcium channels. Science 259:980–984.

Diverse-Pierluissi M, Dunlap K (1993) Distinct, convergent second messenger pathways modulate neuronal calcium currents. Neuron 10:753–760.

Diverse-Pierluissi M, Goldsmith PK, Dunlap K (1995) Transmitter-mediated inhibition of N-type calcium channels in sensory neurons involves multiple GTP-binding proteins and subunits. Neuron 14:191–200.

Ehrlich I, Elmslie KS (1995) Neurotransmitters acting via different G proteins inhibit N-type calcium current by an identical mechanism in rat sympathetic neurons. J Neurophysiol 74:2251–2257.

Elmslie KS, Kammermeier PJ, Jones SW (1992) Calcium current modulation in frog sympathetic neurones: L-current is relatively insensitive to neurotransmitters. J Physiol (Lond) 456:107–123.

Ewald DA, Matthies HJG, Perney TM, Walker MW, Miller RJ (1988) The effect of downregulation of protein kinase C on the inhibitory modulation of dorsal root ganglion neuron Ca^{2+} currents by neuropeptide Y. J Neurosci 8:2447–2451.

Fraser DD, Hoehn K, Weiss S, MacVicar BA (1993) Arachidonic acid inhibits sodium currents and synaptic transmission in cultured striatal neurons. Neuron 11:633–644.

Galvan M, Adams PR (1982) Control of calcium current in rat sympathetic neurons by norepinephrine. Brain Res 244:135–144.

Glass DB, Cheng H-C, Mende-Mueller L, Reed J, Walsh DA (1989) Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. J Biol Chem 264:8802–8810.

Golard A, Siegelbaum SA (1993) Kinetic basis for the voltage-dependent inhibition of N-type calcium current by somatostatin and norepinephrine in chick sympathetic neurons. J Neurosci 13:3884–3894.

Golard A, Role LW, Siegelbaum SA (1993) Protein kinase C blocks somatostatin-induced modulation of calcium current in chick sympathetic neurons. J Neurophysiol 70:1639–1643.

Hescheler J, Schultz G (1993) G-proteins involved in the calcium signalling system. Curr Opinion Neurobiol 3:360–367.

Horn JP, McAfee DA (1980) Alpha-adrenergic inhibition of calcium-dependent potentials in rat sympathetic neurones. J Physiol (Lond) 301:191–204.

House C, Kemp BE (1987) Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. Science 238:1726–1728.

Hug H, Sarre TF (1993) Protein kinase C isoenzymes: divergence in signal transduction? Biochem J 291:329–343.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G protein $\beta\gamma$ subunits. Nature 380:255–258.

Ito M, Tanaka T, Inagaki M, Nakanishi K, Hidaka H (1986) N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide, a novel activator of protein kinase C. Biochemistry 25:4179–4184.

Keyser DO, Alger BE (1990) Arachidonic acid modulates hippocampal calcium current via protein kinase C and oxygen radicals. Neuron 5:545–553.

Kostyuk PG, Veselovsky NS, Fedulova SA (1981) Ionic currents in the somatic membrane of rat dorsal root ganglion neurons-II. Calcium currents. Neuroscience 6:2431–2437.

Lipscombe D, Kongsamut S, Tsien RW (1989) α-Adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium channel gating. Nature 340:639–642.

Liyanage M, Frith D, Livneh E, Stabel S (1992) Protein kinase C group B members PKC-δ, -ϵ, -ζ and PKC-L(η). Comparison of properties of recombinant proteins in vitro and in vivo. Biochem J 283:781–787.

Marchetti C, Brown AM (1988) Protein kinase C activator 1-oleoyl-2-acetyl-sn-glycerol inhibits two types of calcium currents in GH3 cells. Am J Physiol 254:C206–C210.

Mathie A, Bernheim L, Hille B (1992) Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 8:907–914.

Matthies HJG, Palfrey HC, Hirning LD, Miller RJ (1987) Downregulation of protein kinase C in neuronal cells: effects on neurotransmitter release. J Neurosci 7:1198–1206.

Meves H (1994) Modulation of ion channels by arachidonic acid. Prog Neurobiol 43:175–186.

Miller RJ (1990) Receptor-mediated regulation of calcium channels and neurotransmitter release. FASEB J 4:3291–3299.

Mochida S, Kobayashi H (1988) Protein kinase C activators mimic the m_2-muscarinic receptor-mediated effects on the action potential in isolated sympathetic neurons of rabbits. Neurosci Lett 86:201–206.

Nakadate T, Jeng AY, Blumberg PM (1988) Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. Biochem Pharmacol 37:1541–1545.

Nakanishi H, Exton JH (1992) Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J Biol Chem 267:16347–16354.

Nishizuka Y (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.

Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y (1989) Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc Natl Acad Sci USA 86:3099–3103.

Ordway RW, Singer JJ, Walsh JV (1991) Direct regulation of ion channels by fatty acids. Trends Neurosci 14:96–100.

Ozawa K, Szallasi Z, Kazanietz MG, Blumberg PM, Mischak H, Mushinski JF, Beaven MA (1993) Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. J Biol Chem 268:1749–1756.

Plummer MR, Rittenhouse A, Kanevsky M, Hess P (1991) Neurotransmitter modulation of calcium channels in rat sympathetic neurons. J Neurosci 11:2339–2348.

Rane SG, Dunlap K (1986) Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. Proc Natl Acad Sci USA 83:184–188.

Rane SG, Walsh MP, McDonald JR, Dunlap K (1989) Specific inhibitors of protein kinase C block transmitter-induced modulation of sensory neuron calcium current. Neuron 3:239–245.

Sacktor TC, Osten P, Valsamis H, Jiang X, Naik MU, Sublette E (1993) Persistent activation of the ζ isoform of protein kinase C in the maintenance of long-term potentiation. Proc Natl Acad Sci USA 90:8342–8346.

Schofield GG (1990) Norepinephrine blocks a calcium current of adult rat sympathetic neurons via an α_2-adrenoceptor. Eur J Pharmacol 180:37–47.

Schwartz DD, Malik KU (1993) Cyclic AMP modulates but does not mediate the inhibition of [^{3}H]norepinephrine release by activation of α_2-adrenergic receptors in cultured rat ganglion cells. Neuroscience 52:107–113.

Selbie LA, Schmitz-Pfeiffer C, Sheng Y, Biden TJ (1993) Molecular cloning and characterization of PKC 105, an atypical isoform of protein kinase C derived from insulin-secreting cells. J Biol Chem 268:24296–24302.

Shapiro MS, Wollmuth LP, Hille B (1994) Modulation of Ca^{2+} channels by PTX-sensitive G-proteins is blocked by N-ethylmaleimide in rat sympathetic neurons. J Neurosci 14:7109–7116.

Song SY, Saito K, Noguchi K, Konishi S (1991) Adrenergic and cholinergic inhibition of Ca^{2+} channels mediated by different GTP-binding proteins in rat sympathetic neurones. Pflügers Arch 418:592–600.

Werz MA, Macdonald RL (1987) Phorbol esters: voltage-dependent effects on calcium-dependent action potentials of mouse central and peripheral neurons in cell culture. J Neurosci 7:1639–1647.