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Restoration of TEA-Induced Calcium Responses in Fibroblasts from Alzheimer's Disease Patients by a PKC Activator

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Several alterations in fibroblasts of Alzheimer's disease (AD) patients have been described, including alterations in calcium regulation, protein kinase C (PKC), and potassium (K^+) channels. Studies have also found reduced levels of the α isoform of PKC in brains and fibroblasts of AD patients. Since PKC is known to regulate ion channels, we studied K^+ channel activity in fibroblasts from AD patients in the presence of (2*S*, 5*S*)-8-(1-decynyl)benzolactam (BL), a novel activator of PKC with improved selectivity for the α, β, and γ isoforms. We present evidence for restoration of normal K^+ channel function, as measured by TEA-induced $[Ca^{2+}]_i$ elevations, due to activation of PKC by BL. Representative patch-clamp data further substantiate the effect of BL on restoration of 113pS K^+ channel activity. Immunoblotting analyses using an α-isozyme-specific PKC antibody confirm that BL-treated fibroblasts of AD patients show increased PKC activation. The present study suggests that PKC activator-based restoration of K^+ channels may offer another approach to the investigation of AD pathophysiology, which in turn could lead to the development of a useful model for AD therapeutics.

Key Words: **Alzheimer's disease; protein kinase C; calcium; TEA; fibroblast.**

INTRODUCTION

The use of peripheral tissues from Alzheimer's disease (AD) patients and animal neuronal cells permitted the identification of a number of cellular/molecular alterations that may be the reflection of comparable processes in the AD brain and thus, of pathophysiological relevance (Baker *et al.,* 1988; Scott, 1993; Huang, 1994; Scheuner *et al.,* 1996; Etcheberrigaray & Alkon, 1997; Gasparini *et al.,* 1997). Alterations of potassium channel function have been identified in fibroblasts (Etcheberrigaray *et al.,* 1993) and in blood cells (Bondy *et al.,* 1996) obtained from AD patients. In addition, it was shown that β-amyloid, widely accepted as a major player in AD pathophysiology (Gandy & Greengard, 1994; Selkoe, 1994; Yankner, 1996), was capable of inducing an AD-like K^+ channel alteration in control fibroblasts (Etcheberrigaray *et al.,* 1994). Similar or comparable effects of β-amyloid on K^+ channels have been reported in neurons from laboratory animals (Good *et al.,* 1996; also for a review see Fraser *et al.,* 1997). An earlier observation of hippocampal alterations of apamin-sensitive K^+ channels in AD brains (as measured by apamin binding) provides additional support for the suggestion that K^+ channels may be pathophysiologically relevant in AD (Ikeda *et al.,* 1991). Furthermore, protein kinase C (PKC) exhibits parallel changes in peripheral and brain tissues of AD patients. The levels and/or activity of this enzyme(s) were reduced in brains and fibroblasts from AD patients (Cole *et al.,* 1988; Van Huynh *et al.,* 1989; Govoni *et al.,* 1993; Wang *et al.,* 1994). Studies using immuno-

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blotting analyses have revealed that of the various PKC isozymes, primarily the α isoform was significantly reduced in fibroblasts (Govoni *et al.,* 1996), while both α and β isoforms are reduced in brains of AD patients (Shimohama *et al.,* 1993; Masliah *et al.,* 1990). These brain PKC alterations might be an early event in the disease process (Masliah *et al.,* 1991). It is also interesting to note that PKC activation appears to favor nonamyloidogenic processing of the amyloid precursor protein (APP; Buxbaum *et al.,* 1990; Gillespie *et al.,* 1992; Selkoe, 1994; Gandy & Greengard, 1994; Bergamashi *et al.,* 1995; Desdouits *et al.,* 1996; Efhimiopoulus *et al.,* 1996). Thus, both PKC and K^+ channel alterations appear to coexist in AD, with peripheral and brain expression in AD.

Therefore, we decided to investigate a potential link between PKC and K^+ channel alterations. Because PKC is known to regulate ion channels, including K^+ channels (e.g., see Alkon *et al.,* 1988; Covarrubias *et al.,* 1994; Hu *et al.,* 1996), we hypothesize that a defective PKC may lead to defective K^+ channels. To test this hypothesis we used AD fibroblasts in which both K^+ channels and PKC defects have been independently demonstrated (Etcheberrigaray *et al.,* 1993; Govoni *et al.,* 1993, 1996). Fibroblasts with known dysfunctional K^+ channels were treated with PKC activators and restoration of channel activity was monitored as presence / absence of TEA-induced calcium elevations. An assay based on tetraethylammonium chloride (TEA)-induced $[Ca^{2+}]_i$ elevation was used because it has been shown to depend on functional 113pS K^+ channels that are susceptible to TEA blockade (Etcheberrigaray *et al.,* 1993, 1994; Hirashima *et al.,* 1996). Thus, TEA-induced $[Ca^{2+}]_i$ elevations and K^+ channel activity are primarily observed in fibroblasts from control individuals while being virtually absent in fibroblasts from AD patients (Etcheberrigaray *et al.,* 1993; Hirashima *et al.,* 1996). Here we show that the use of a potent novel PKC activator, benzolactam (BL), restores the responsiveness of AD fibroblast cell lines to the TEA challenge. We also present immunoblot evidence that this restoration might be related to a preferential participation of the α isoform since BL shows improved selectivity for this isozyme that is defective in AD fibroblasts.

MATERIALS AND METHODS

Cell Culture

Cultured skin fibroblasts were obtained from the Coriell Cell Repositories and grown using the general guidelines established for their culture with slight modifications (Cristofalo & Carpentier, 1988; Hirashima *et al.,* 1996). The culture medium in which cells were grown was Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Biofluids, Inc.). Seven age-matched controls (AC) (AG06241, AG07141, AG05266, AG09878, AG04560, AG06010, and AG08044), three sporadic AD (SAD) cases (AG06263, AG07375, and AG07377), and four familial AD (FAD) cases—including Canadian family 964 (Nee *et al.,* 1983) (AG06848, AG08170), Italian family 1079 (AG07872), and pedigree 747 (AG04401)—were tested in the calcium-imaging experiments. Mutations in the PS1 gene are expressed in both the Canadian (an Ala → Glu substitution in codon 246; PS1A246E) and the Italian (an Met → Leu substitution at codon 146; PS1M146L) family cell lines. For immunoblotting experiments, the same set of seven AC and AD cell lines tested in the imaging experiments were used. Detailed information about these cell lines can be obtained elsewhere (Nee *et al.,* 1983; National Institute of Aging, 1994).

PKC Activators

The different tissue distributions, the apparently distinctive roles of different isozymes, and the differential involvement in pathology make it important to use pharmacological tools that are capable of preferentially targeting specific isozymes (Kozikowski *et al.,* 1997; Hofmann, 1997). Recent research in the medicinal chemistry field has resulted in the development of non-tumor-promoter PKC activators that target with greater specificity the α and β isoforms (Kozikowski *et al.,* 1997). One such compound is (2*S*,5*S*)-8-(1-decynyl)-benzolactam (benzolactam), a derivative of the natural product indolactam V. Indolactam V was first isolated from the mycelia of *Streptomyces mediocidicus* and is a member of the teleocidin family. These compounds, like the phorbols, compete for the regulatory domain of PKC and engage in very specific hydrogen bond interactions within this site. Additional information on the organic chemistry and molecular modeling of this compound can be found elsewhere (Kozikowski *et al.,* 1997). For comparison purposes, we also used the nonselective well-known PKC activator, phorbol 12,13-dibutyrate.

Calcium Imaging

Round, 25-mm glass coverslips were flame-polished and placed inside 35-mm Nunc petri dishes. Cells were

seeded (ca. 10–15 cells / mm^2) in these petri dishes and used when cell density was equivalent for all cell lines (ca. 180 cells / mm^2). General aspects of the calcium-imaging technique and the theoretical foundation are described elsewhere (Thomas *et al.*, 1991). Briefly, culture medium was removed and cells were washed at least three times with basal salt solution (BSS; in mM: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, Hepes 10, glucose 5, pH 7.4). The fluorescent probe was loaded by incubating cells in 1 μM fura 2-AM (Molecular Probes) in 1 ml BSS at room temperature (RT). One hour later, cells were washed thoroughly with BSS and 1 ml of fresh BSS was added for $[Ca^{2+}]_i$ baseline measurements. Fluorescent images at 334 and 380 nm were acquired so that the rate of ratios was approx 1 / s to about 300 s with a Zeiss-Attofluor Ratio Arc Imaging System (Zeiss). A 40× Zeiss Fluor (oil immersion) objective lens was used.

Treatment

Cells from all treatment groups were tested with 100 mM TEA (Sigma) by applying 3 ml of TEA-modified BSS (in mM: TEA 133.3, NaCl 6.7, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, Hepes 10, glucose 5, pH 7.4) to the dish. TEA was dissolved in BSS and the solution was accordingly modified to prevent osmolarity changes (Hirashima *et al.*, 1996). A group of cells was treated with the PKC activator benzolactam prior to the TEA challenge. BL was dissolved in dimethyl sulfoxide (DMSO; Sigma) to achieve a final testing concentration of 50 nM when 1 ml of the dissolved BL was added to the dish 1 min prior to TEA application. A second group of cells was incubated with the nonselective PKC activator, phorbol 12,13 dibutyrate (PDBu; Calbiochem) at 50 nM for 45 min by adding the compound (dissolved in DMSO) to the dish 15 min after the initiation of the dye-loading phase. A preliminary study also included 1 and 15 min incubation with PDBu. The control group of cells included cells which received no treatment prior to TEA challenge (untreated), cells pretreated with 50 nM DMSO for 1 min, and cells incubated with 50 nM 4α-phorbol (4α-PHR; Calbiochem), an inactive form of phorbol ester (negative control), for 45 min. Seven to ten petri dishes of cells were tested from each cell line for each experimental condition. In almost all the cell lines, a minimum of 100 cells were tested per treatment per cell line (total 9231 cells). There were only three AC cell lines for which the number of cells in a couple of treatment groups was above 65 but below 100. Experiments for each cell line were repeated on at least one separate occasion (minimum 1-week interval). Response in a given cell was characterized as a double-fold increase in baseline $[Ca^{2+}]_i$ (75–90 nM) based on previously established criteria (Etcheberrigaray *et al.*, 1993; Hirashima *et al.*, 1996).

Electrophysiology and Single-Channel Analysis

Patch-clamp experiments were performed on 13 separate replicate representative experiments in a well-characterized AD cell line (AG06848) which is normally silent for the 113pS TEA-sensitive K^+ channel (Etcheberrigaray *et al.*, 1993). The experiment was performed at room temperature (21–23°C) following standard procedures (Sakmann & Neher, 1983). The culture medium was replaced with the following solution (mM), NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Hepes (NaOH) 10, pH 7.4, prior to recording. Benzolactam was added to a dish after ensuring that the patch is silent for the 113pS K^+ channel. The recording electrodes, filled with a high-K^+ solution (mM), KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, Hepes (NaOH) 10, pH 7.4, were made from Blu Tip capillary tubes (i.d. 1.1–1.2 mm; Oxford Labware) by using a BB-CH-PC Mecanex puller to obtain pipette resistances that were ~6 MΩ. An Axopatch-1C amplifier was used to obtain records and a JVC Vetter PCM videorecorder was used to store the data on tape. The data were acquired and analyzed on a personal computer with a pClamp suite of programs by transferring the information via an Axolab interface from the amplifier. All three components—amplifier, interface, and software—were obtained from Axon Instruments (Foster City, CA).

Immunoblot Assay

Immunoblot experiments were conducted using well-established procedures (Dunbar, 1994). Cells were grown to confluency (~90%) in T-75 flasks. Levels of α isozyme in response to treatment with 50 nM BL for 1 and 15 min or 50 nM PDBu for 1 and 45 min were quantified using procedures slightly modified from that established by Racchi *et al.* (1994). Fibroblasts were washed twice with ice-cold PBS, scraped in PBS, and collected by low-speed centrifugation. The pellets were resuspended in the following homogenization buffer: 20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 2 mM

EGTA, 5 mM DTT, 0.32 M sucrose, 2 mM PMSF, 25 μg/ml aprotinin, and 20 μg/ml leupeptin. Homogenates were immersed in liquid nitrogen, thawed, and centrifuged at 12,000*g* for 20 min, and the supernatants were used as the cytosolic fraction. The pellets were homogenized in the same buffer containing 1.0% Triton X-100, incubated in ice for 45 min, and centrifuged at 12,000*g* for 30 min. The supernatant from this batch was used as the membranous fraction. After protein determination, 20 μg of protein was diluted in 2 × electrophoresis sample buffer (Novex), boiled for 5 min, run on 12% SDS–PAGE, and transferred electrophoretically to nitrocellulose membrane. The membrane was saturated with Blotto blocker (Pierce) by incubating it at RT for an hour. The primary antibody for PKC α isoform (Transduction Laboratories) was diluted (1:1000) in blocking solution and incubated with the membrane for 1 h at RT. After incubation with the secondary antibody, alkaline phosphatase anti-mouse IgG (Vector Laboratories), the membrane was developed using an alkaline phosphatase kit (Vector Laboratories) as per the manufacturer's instructions. The conditions for developing the membranes were kept as similar as possible. After a digital image of each nitrocellulose membrane was procured (Lightools Research Speedlight Gel Documentation System), although the OD value (by performing densitometric analyses; NIH Image version 1.6) for each signal was obtained, the ratio of membrane to particulate PKC fraction was used when comparing among experiments to avoid idiosyncratic variability. The assay was repeated at least twice for all cell lines except two AC (AG05266, AG09878) and two AD (AG07872, AG07375) cell lines. The data from each replicate were pooled for each cell line and averaged across cell lines for each treatment group.

Statistical Analyses

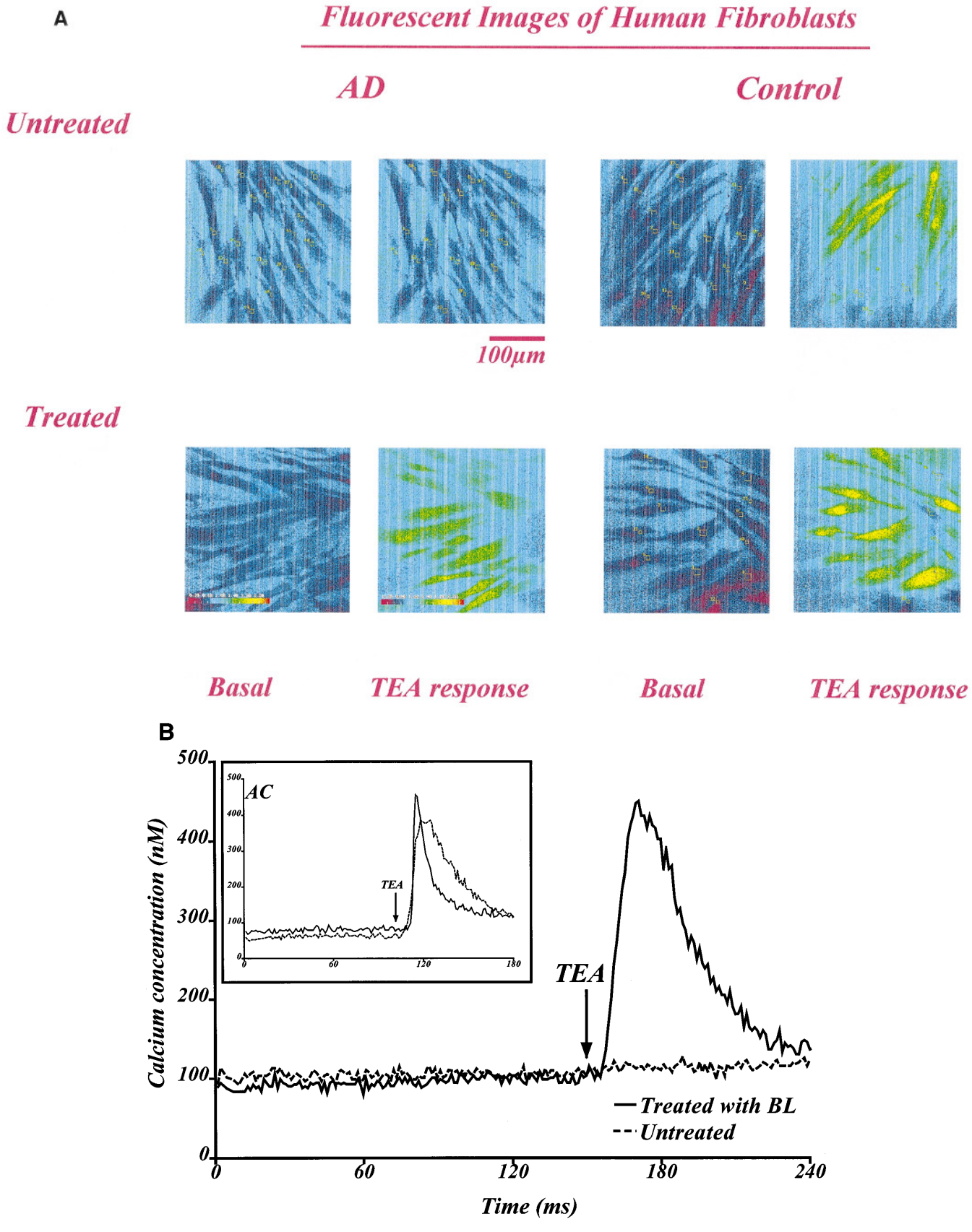
Data were pooled from FAD and SAD cell lines since initial analyses revealed no significant differences in response patterns between the two types of cell lines in both imaging and immunoblotting studies. All imaging and immunoblot data analyses were performed using the two-tailed *t* test (Remington & Schork, 1995) and, in cases in which the variances were different, using the Welch correction (Motulsky, 1995) in Graph Pad Prism (version 2) for IBM. The sole exception was the one-tailed *t* test statistical comparison to evaluate whether the level of PKC immunoreactivity after 1 min treatment with BL is stronger than the 1 min treatment with PDBu.

RESULTS

Pseudocolor fluorescent ratio images of basal and TEA-induced calcium levels in fibroblasts excited by 334 (numerator) and 380 nm (denominator) UV light are shown in Fig. 1A. Pretreatment of AD fibroblasts with benzolactam (Fig. 1B, solid lines) restores the typical increase in intracellular calcium following TEA treatment in contrast to the untreated AD cells (broken line). On the other hand, $[Ca^{2+}]_i$ elevations occurred in both untreated and BL-pretreated AC cells, due to TEA-induced depolarization by blockade of 113pS K^+ channels (Fig. 1, inset).

Following TEA challenge, the percentage of cells responding to TEA was measured and averaged across cell lines for AC and AD cases (Fig. 2). Upregulating PKC (predominantly the α isoform) in AD fibroblasts restored TEA response. Treatment of AD cell lines with 50 nM BL for 1 min (black bar) significantly increased the percentage of cells responding to TEA compared to the untreated (open bar; $t = 2.99$, $P = 0.017$) and DMSO-treated (light gray bar; $t = 3.60$, $P = 0.0113$) controls. In contrast, AD cells incubated with 50 nM nonselective PKC activator, PDBu, for 45 min did not differ in their TEA response (hatched bar) from the 4α-PHR-treated cells (dark gray bar; $t = 0.22$, $P = 0.83$). Shorter incubation times (1 and 15 min) with PDBu were also without effect (data not shown). Typical TEA response was observed in all AC cells irrespective of treatment (Fig. 2, inset). Percentage of response in BL-treated cells did not differ from untreated ($t = 0.02$, $P = 0.98$) or DMSO-treated ($t = 0.88$, $P = 0.39$) AC cells. Likewise, percent-

FIG. 1. (A) Fluorescent images of human fibroblasts in culture at subconfluent levels. Pseudocolor ratio images represent calcium levels as seen when cells are excited by 334 nm (numerator) and 380 nm (denominator) UV light. Brighter ratio images depict intracellular increase in calcium levels. While there is no difference in calcium levels in untreated AD cells before and after TEA application, AD cells pretreated for 1 min with benzolactam (BL) respond to TEA with an increase in intracellular Ca^{2+} from basal levels. On the other hand, there is an increase in Ca^{2+} in both untreated and BL-pretreated AC cells following TEA challenge. (B) TEA response profile in AD cells. Lack of intracellular Ca^{2+} elevation following TEA application in untreated AD cells is reversed in BL-pretreated AD cells. Inset: TEA application results in increase in Ca^{2+} levels in both untreated and BL-pretreated AC cells. Lines represent the same treatments as in B.



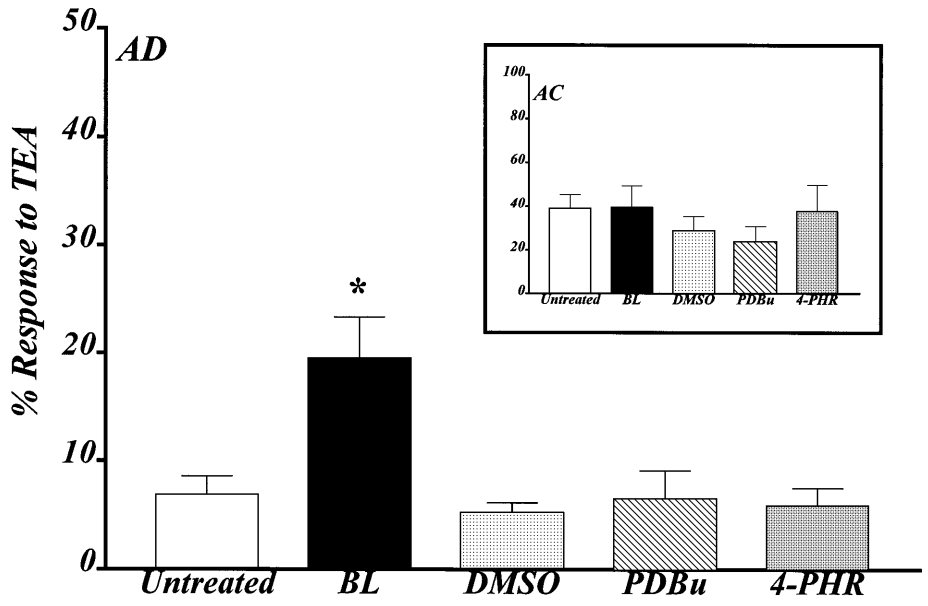


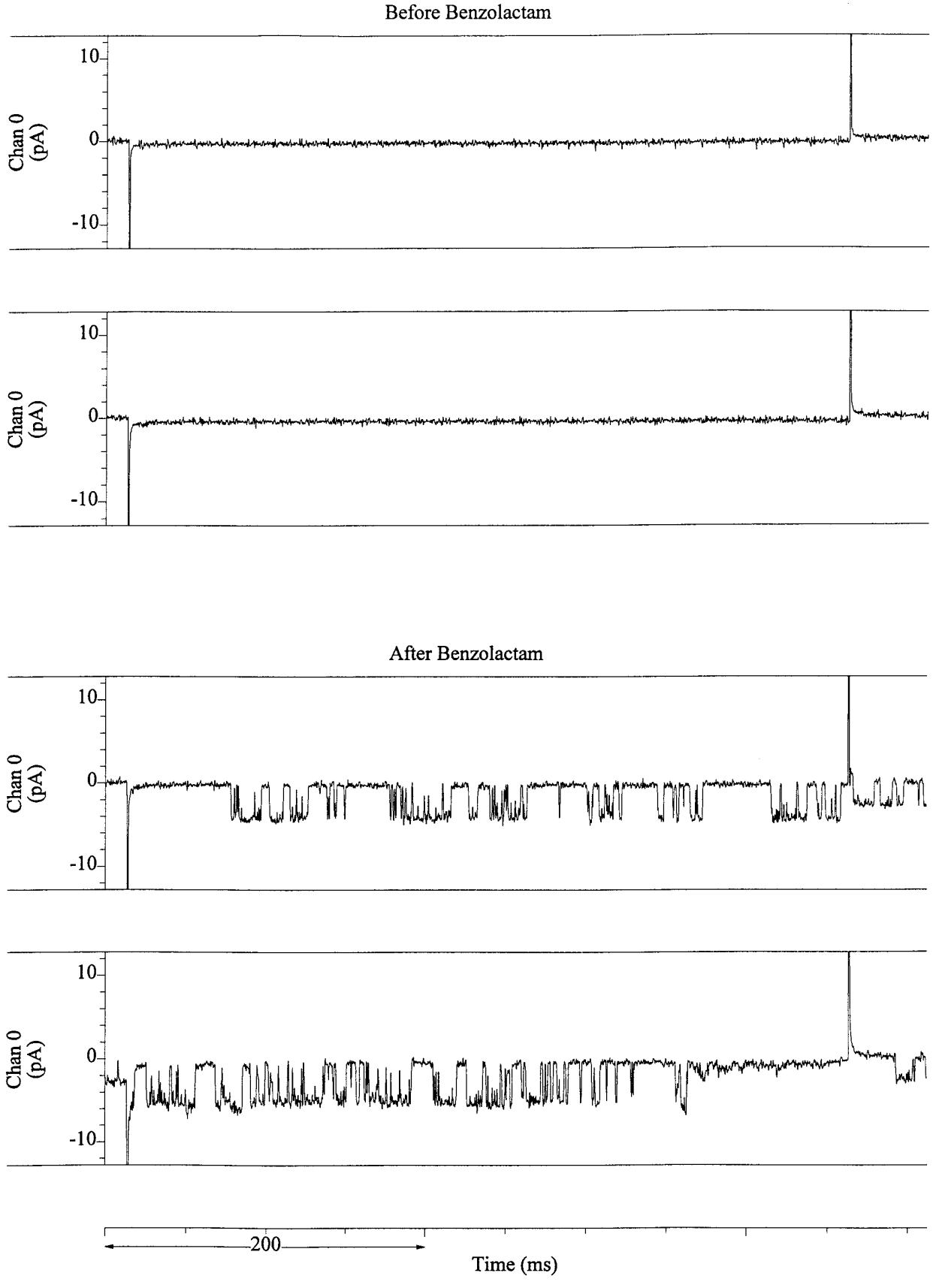
FIG. 2. Calcium elevations in response to TEA expressed as percentage of responding cells. Each of the cell lines received one of the following treatments—none (open bar; $N = 1067$), 1 min pretreatment with either BL (black bar; $N = 1038$) or DMSO (light gray bar; $N = 974$), or 45 min incubation with either PDBu (hatched bar; $N = 1047$) or 4α-phorbol (dark gray bar; $N = 1034$). Data from each replicate within each cell line were pooled and averaged (means + SE) across the seven AD cell lines for each treatment. Benzolactam pretreatment significantly increased the TEA responsiveness compared to untreated or DMSO-treated controls (*$P < 0.05$ in either case). In contrast, there was no difference between PDBu and 4α-PHR-pretreated AD cells (ns). Two-tailed t test was used to make statistical comparisons. Inset: Normal response to TEA in AC cells irrespective of treatment—none (open bar; $N = 887$), pretreatment with BL (black bar; $N = 824$), DMSO (light gray bar; $N = 754$), PDBu (hatched bar; $N = 794$), or 4α-phorbol (dark gray bar; $N = 812$). Neither BL-treated AC cells differed from untreated or DMSO-treated cells in percentage of cells responding to TEA nor PDBu-treated cells from their respective control, the 4-PHR-treated AC cells. Percentage of cells responding to TEA is significantly higher in untreated AC versus untreated AD cells ($P < 0.005$).

age of PDBu-treated cells responding to TEA was not different from the 4α-PHR-treated ($t = 1.01$, $P = 0.33$) AC cells. The significantly higher percentage of ''responsive'' untreated AC (39.19 ± 6.27) versus the untreated AD (6.31 ± 1.75) cells (open bars in main graph and inset; $t = 4.97$, $P = 0.0025$) is consistent with previous findings (Etcheberrigaray *et al.*, 1993; Hirashima *et al.*, 1996).

Patch-clamp analyses revealed that none of the 13 recordings showed K^+ channel activity before application of BL to the medium (see Fig. 3, top two panels). However, after 1 min BL treatment, 7 of the same 13 cells showed unambiguous 113pS K^+ channel activity (see Fig. 3, bottom two panels). The 54% K^+ channel activity after BL activation in these AD cells is comparable to that observed in control cells in a previous study (Etcheberrigaray *et al.*, 1993).

Immunoblot assay provides an additional support for our hypothesis that PKC is involved in the restoration of TEA response (Figs. 4A and 4B). Compared to the basal ratios (Fig. 4B, open bar), treatment of AD cell lines with 50 nM BL for 1 min (Fig. 4B, black bar) revealed a pronounced redistribution of the α isoform of the enzyme from cytosol to membrane ($t = 3.67$; $P < 0.05$). A longer incubation time (15 min; data not shown) with BL further increased the particulate/soluble ratios (ratio = 1.156; $t = 4.48$; $P < 0.005$) compared to basal levels, indicating lack of downregulation within the time frame studied. Although 1 min treatment with 50 nM PDBu (Fig. 4B, gray bar) yielded a significant change from the basal ratio level ($t = 3.67$; $P < 0.005$) in AD cells, it was not as effective as the 1 min treatment with 50 nM BL (black vs gray bar, $t = 2.27$, $P < 0.05$). Longer incubation (45 min) with

FIG. 3. Cell-attached recordings in human fibroblasts. Representative traces obtained from cell line AG06848 (FAD). The top two traces correspond to a silent patch at 0 and +20-mV pipette potentials. Distinct channel activity (bottom two traces) was observed in the same patch after adding a PKC activator, benzolactam, to the dish.



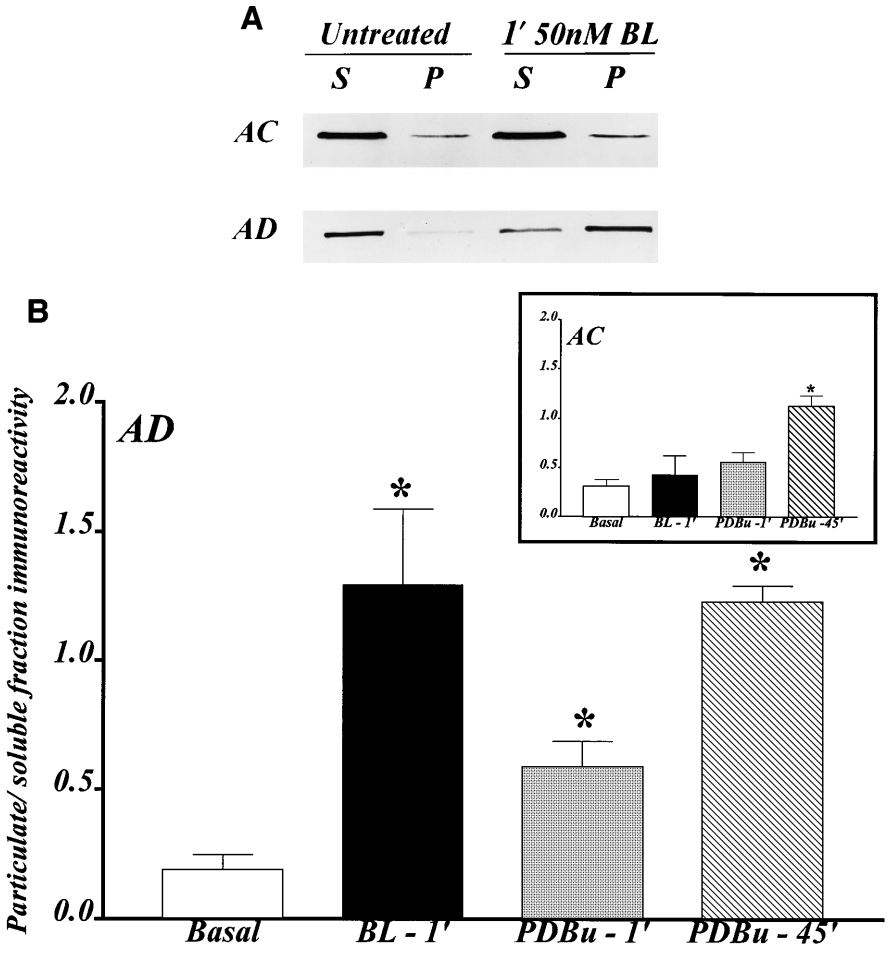


FIG. 4. Translocation of PKCα induced by benzolactam. (A) Representative immunoblots from an AC and an AD cell line treated with BL for 1 min. (B) PKC redistribution from cytosol (soluble fraction) to membrane (particulate fraction) expressed as ratio of particulate to soluble fraction immunoreactivity measured by densitometric analyses in seven AD cell lines. Ratio values for untreated, 1 min treatment with BL or PDBu, and 45 min pretreatment with PDBu are represented by open, black, gray, and hatched bars, respectively. Data from each replicate were pooled within each cell line and averaged (means + SE) across cell lines. Benzolactam treatment of AD cells significantly increased the level of immunoreactivity from basal levels (*$P < 0.05$). Incubation with PDBu for 1 or 45 min also enhanced the level of enzyme activity from basal level (*$P < 0.05$). Two-tailed t test was used for statistical comparisons. Inset: PKC immunoreactivity in AC cells. Data were averaged across seven AC cell lines after pooling replicates within each cell line. Bars represent the same treatments as in the main graph. Benzolactam or PDBu treatment for 1 min did not have an effect on immunoreactivity of the enzyme in AC cells. However, incubation with PDBu for 45 min significantly increased the level of enzyme activity compared to the basal level (*$P < 0.05$; two-tailed t test).

PDBu (Fig. 4B, hatched bar) resulted in levels of PKC translocation comparable to that observed with 1 min BL treatment in AD cells ($t = 10.52$; $P < 0.001$).

In AC cells, effect of BL on enzyme translocation was significant only after 15 min treatment with the PKC activator (data not shown; ratio = 1.72; $t = 10.37$; $P < 0.01$) and not after 1 min treatment (Fig. 4, inset, black bar, $t = 0.56$, ns) compared to basal levels (open bar). Likewise, long-term incubation (45 min) with PDBu had an effect on redistribution of the enzyme from cytosol to membrane (hatched bar, $t = 6.78$, $P < 0.001$). There was no effect of 1 min incubation with PDBu (gray bar, $t = 2.16$, ns) on PKC translocation in AC cells.

DISCUSSION

These results provide evidence for a significant and perhaps primary role for PKC in AD and suggest a mechanistic explanation for the various molecular and cellular defects observed in fibroblasts from AD patients. Restoration of the TEA-induced $[Ca^{2+}]_i$ re-

sponses in BL-treated AD cells indirectly implies restoration of their K^+ channel function. Patch-clamp data strongly support the conclusions from imaging studies in a representative sample obtained from a well-characterized AD cell line. BL was chosen since it has been shown to have improved isozyme specificity for the α and β isozymes and, to a lesser extent, for the γ isoform (Kozikowski *et al.,* 1997). This improved selectivity is of particular relevance in AD fibroblasts, in which only the α isoform appears to be affected. In fact, the use of a compound that primarily targets this isoform proved to be a successful strategy to restore K^+ channel function in fibroblasts. Moreover, this effect was not seen in cells treated with PDBu, a nonspecific and—at equimolar concentrations—less potent activator of PKC. Although BL also exhibits high selectivity for the β isoform, the participation of this isozyme in restoring K^+ channel function can be ruled out since it is not expressed in fibroblasts (Racchi *et al.,* 1994). We confirmed this observation in a subsample of the cell lines used here (not shown). The γ isoform is also not expressed in fibroblasts (Racchi *et al.,* 1994). The isoforms δ and ϵ are detected in fibroblasts (Racchi *et al.,* 1994) but the BL affinity for them is at least 10-fold lower than for α (Kozikowski *et al.,* 1997). Furthermore, they do not appear to be altered in AD fibroblasts (Govoni *et al.,* 1996).

The percentage of untreated AC cells responding to TEA (39.19%) is comparable to response levels reported in a previous study (Etcheberrigaray *et al.,* 1993). Interestingly, treating AD cells with BL enhanced the level of TEA responsiveness (19.48%) to levels reported for AC cells in previous studies (Hirashima *et al.,* 1996). Lack of such enhancement in BL-treated AC cells is, perhaps, due to the system reaching a steady-state equilibrium of activity in these cells.

The immunoblot assay, under conditions approximating the imaging studies as closely as possible, provided an independent method of assessing that PKC is, indeed, involved in the restoration of the normal K^+ channel phenotype. At equimolar concentrations, the effect of BL treatment was clearly stronger at 1 min compared to the effect of PDBu at 1 min for both imaging and immunoblot studies. However, PDBu was able to induce a noticeable change in PKC translocation but not in TEA-induced Ca^{2+} elevation at a longer (45 min) time interval. Perhaps this singular disparity between imaging and immunoblot results is due to the different dynamics between activation of PKC and TEA-induced Ca^{2+} elevations. Although our experimental design did not demonstrate a *direct* effect of PKC on the channels, the PKC changes are observed within a time frame and under experimental conditions compatible with a causal link. Whether PKC acts directly on the channel or via an intermediary remains to be elucidated.

It is also interesting to consider the data in relation to APP metabolism and the effects of its subproducts. Studies have demonstrated that PKC activation increases the amount or ratio of nonamyloidogenic (soluble APP, presumably product of the α secretase) vs amyloidogenic (Aβ1-40 and/or Aβ1-42) secreted fragments (Buxbaum *et al.,* 1990; Gillespie *et al.,* 1992; Selkoe, 1994). We could speculate that AD cells with low PKC would have an impaired secretion of sAPP and/or have increased proportion of amyloidogenic fragments. Indeed, there is evidence that some AD cell lines exhibit both defective PKC and impaired sAPP secretion (Bergamaschi *et al.,* 1995; Govoni *et al.,* 1996). A subset of those cells was shown to have defective TEA responses (i.e., defective K^+ channels; Hirashima *et al.,* 1996). Moreover, at least three of the FAD cell lines used in this study are from individuals demonstrated to carry presenilin 1 mutations (St. George-Hyslop *et al.,* 1992; Sherrington *et al.,* 1995; Tanzi *et al.,* 1996) and also to have increased levels of $\beta\text{-amyloid}_{1-42}$ (Hardy, 1997; Scheuner *et al.,* 1996). In addition, β-amyloid has been shown to induce an AD-like K^+ channel defect in fibroblasts (Etcheberrigaray *et al.,* 1994) and to block K^+ currents in cultured neurons (Good *et al.,* 1996). Therefore, we suggest a mechanistic link such that an isozyme-specific PKC defect may lead to abnormal APP processing that, among other possible deleterious effects, alters K^+ channel function. Recent preliminary data also suggest that, perhaps in a vicious cyclical manner, β-amyloid in turn causes reductions of α-PKC (Favit *et al.,* 1997).

In summary, our data suggest that the strategy to upregulate PKC function targeting specific isozymes restores normal K^+ channel function. The restoration of K^+ function may have direct consequences in terms of reducing the progression of the pathological process. These studies and such a fibroblast model could be expanded and used as tools to monitor the effect of compounds (BL, for example) that alter potential underlying pathological processes. Such compounds could then be used as bases for rational design of pharmacological agents for this disorder.

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