The Effects of 2-Aminoethoxydiphenyl Borate and Diphenylboronic Anhydride on Gap Junctions Composed of Connexin43 in TM4 Sertoli Cells

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2-Aminoethoxydiphenyl borate (2-APB) has recently been demonstrated to inhibit gap junction (GJ) channels, whereas the underlying mechanisms are still unknown. Using mouse TM4 Sertoli cell which expresses connexin43 (Cx43), we explored the effects of 2-APB and its analogues on dye-coupling through junctional channels formed by Cx43 and on expression of Cx43. Exposure of the cells to 2-APB (1—50 μM) and one of its analogues diphenylboronic anhydride (DPBA) (1—30 μM) for 4 h leads to a significant decrease in dye coupling of GJ in a concentration-dependent manner. The inhibitory effects of 2-APB and DPBA are reversible since decreased GJ coupling resumes after the two compounds are washed out. The disfunction of GJ induced by 2-APB and DPBA is associated with a decrease in total amount of Cx43 protein and number of GJs on the cell membrane. 2-APB and DPBA do not alter Cx43 phosphorylation state and the level of Cx43 mRNA expression. The loss of Cx43 protein is prevented by either lysosomal or proteasomal inhibitor, suggesting that the decrease in Cx43 results from a 2-APB or DPBA-enhanced degradation of Cx43. The present results indicate that 2-APB and DPBA inhibit GJ communication through decreasing Cx43 expression in TM4 cells.

Key words 2-aminoethoxydiphenyl borate; diphenylboronic anhydride; gap junction; connexin43; TM4 cell

Connexin (Cx) channels, which compose vertebrate gap junctions (GJs), are transmembrane channels that directly link the cytoplasm of neighbouring cells, thereby allowing for the passive transfer of small cytoplasmic signaling molecules. There are approximately 21 isoforms of Cx protein, each of which forms channels with distinct regulation and permeability. The intercellular signaling mediated by Cx channels is important: almost every functional deletion of a Cx isoform produces a distinct pathology.

The Cxs are differentially expressed in a variety of tissues, which is generally believed to reflect cell-specific regulation of junctional coupling and functional demands for GJs in different cell types. Among all Cxs, Cx43 is the predominant Cx isoform in many cell types and tissues. In testis, Cx43 is the most abundant and ubiquitously distributed GJ protein. Cx43 is localized between Sertoli cells, and between Sertoli and germ cells. It was reported that spermatogenesis required direct intercellular communication between Sertoli cells. In cardiovascular system, Cx43 channels play essential roles in mediating the spread of the electrical impulse and contribute to the coordinate activities between cardiocytes. Cx43 is also primarily expressed in astrocytes where they impact the blood–brain barrier, playing a crucial role in maintaining the homeostasis of the extracellular milieu of neurons. In addition, the important role of Cx43 in physiology and pathology has also been elucidated in many processes, such as wound healing and remodelling process after injury in skin, erectile response and cancer development.

2-Aminoethoxydiphenyl borate (2-APB), an extensively used agent to inhibit the release of intracellular Ca2+, is originally characterized as a cell-permeable inhibitor of inositol trisphosphate (IP3) receptors. However, numerous studies have demonstrated that 2-APB affects other channels and transporters including inhibition of some GJ channels.

Pan, et al. screened a series of potential GJ antagonists by evaluating their effects on dye coupling and found 2-APB being one of the four most potent GJ blockers. We previously reported that 2-APB inhibited GJ channels composed of Cx26 and/or Cx32 by acting on Cx channel directly. The direct and indirect evidences strongly support a potential role of 2-APB as a novel and efficient inhibitor of GJ channels. However, 2-APB was mostly used by electrical measurement of coupling with the treatment time scale within minutes, very little is known about its effect on permeability of Cx43 channel and the underlying mechanisms still remain to be elucidated.

In the present study, we explored the effects of 2-APB and its analogues on dye coupling through junctional channels formed by Cx43 and on expression of Cx43 in TM4 Sertoli cell, an immortalized non-tumorogenic cell line derived from mouse testis. The data presented here show that 2-APB and its analogue diphenylboronic anhydride (DPBA) reduce dye coupling of GJ composed of Cx43 reversibly in a concentration-dependent manner. And this inhibitory effect of 2-APB on DPBA on Cx43 channel results from, at least in part, the decrease in Cx43 protein level.

MATERIALS AND METHODS

Drugs, Antibodies and Reagents 2-Aminoethoxydiphenyl borate (2-APB), diphenylboronic anhydride (DPBA), 2,2-diphenyltetrahydrofuran (DPTTF), diphenyldramine (DPDM) and phenytoin (PHT) were from Calbiochem (San Diego, CA, U.S.A.). Dimethyl sulfoxide (DMSO), 12-O-tetradecanoylphorbol 13-acetate (TPA), leupeptin (acytel-leu-leu-arg-al), ALLN (N-acetyl-leu-leu-norleucinal) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO, U.S.A.). Trizol, cell labeling dyes CM-Dil and calcein-
AM (acetoxyethyl ester), and fluorescein isothiocyanate (FITC)-anti-mouse immunoglobulin G (IgG) were from Invitrogen (Carlsbad, CA, U.S.A.). The primary antibody mouse monoclonal anti-Cx43 IgG for western blotting or immunofluorescent analysis was from Sigma. Alkaline phosphatase-linked secondary antibodies were from Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.). Cell culture reagents were obtained from Invitrogen. All other reagents were from Sigma unless stated otherwise.

**Cell Lines and Cell Culture** The mouse TM4 Sertoli cell line was obtained from American Tissue Culture Collection (ATCC). Cells were cultured in 37 °C with Dulbecco’s modified Eagle’s medium: F12 (DEME: F12) supplemented with 15 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) buffer, 5% horse serum, 2.5% fetal calf serum and 1% penicillin-streptomycin. The medium was changed every 2 d. However, for the studies involving the drug treatment for 48 h in MTT assay, the outer medium was exchanged every 12 h to exclude the possibility of depletion of the drug in the long-term exposure.

**MTT Assay** Cell viability was determined by the MTT colorimetric assay capable of detecting viable cells by the reduction of the yellow tetrazolium salt to purple formazan. To rule out the possibility that the cytotoxic effect may not be observed by a short time treatment, TM4 cells were seeded at 0.5×10^5 cells/well in 96 well plates for 1 d and then exposed to various concentrations of 2-ABP, DPBA, DPTTF, DPDM and PHT for 48 h. Cells incubated with DMSO at the same concentration (always less than 0.1% v/v) were used as a control. MTT (5 mg/ml in phosphate buffered saline (PBS)) was then added to each well, and the dishes were incubated at 37 °C for 4 h, the medium containing MTT was then removed. The formazan crystals in the viable cells were solubilized with 100 μl DMSO and the absorbance at 490 nm of each well was read using a microplate enzyme-linked immunosorbent assay (ELISA) reader (MRX II, Dynex Technologies, Chantilly, VA, U.S.A.). All experiments were performed at least three times, with six wells for each concentration of the tested compounds (n=6 per experiment). Final results were the average of three independent experiments. The cell viability was calculated as follows: (OD of experimental group−OD of blank group)/(OD of control group−OD of blank group).

**“Parachute” Dye-Coupling Assay** This assay for gap junctional intercellular communication (GJIC) determination was performed as described previously.27,28 Donor and receiver cells were grown to confluence. The donor cells were double-labeled with 5 μM CM-DiI, a membrane dye that does not spread to coupling cells, and 5 μM calcein-acetoxyethyl ester, which is converted into calcein in intracellular plasma to be GJ-permeable, for 30 min at 37 °C. The donor cells were then trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The donor cells were allowed to attach to the monolayer of receiver cells and form GJs for 4 h at 37 °C and then examined with a fluorescence microscope. For each experimental condition, the average number of receiver cells containing calcein per donor cell of 10–20 fluorescent areas was determined and normalized to that of control cultures.

**Western Blotting** For western blotting analysis, cells were lysed in lysis buffer [50 mM NaH2PO4, 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol bis(2-aminoethylther)-N,N,N’,N’-tetraacetic acid (EGTA), 80 mM n-octyl β-D-glucopyranoside, 1 mM β-mercaptoethanol, 0.5 mM di-isopropyl fluorophosphate, (pH 7.5)] at 4 °C, followed by a brief sonication. The suspension was then centrifuged at 12000 rps for 30 min at 4 °C and proteins from the supernatant were extracted. Protein determination was performed using a protein assay kit (Bio-Rad Chemical Co.). Samples (20 μg) from cells were applied to sodium dodecyl sulfate (SDS)-polyacrylamide gels of 15% or 8% (w/v) acrylamide, followed by electrophoresis and blotting. Immunodetections were performed using the following antibodies at dilution recommended by the suppliers respectively: mouse monoclonal anti-Cx43 IgG as primary antibody at 1:4000 dilution; alkaline phosphatase-conjugated goat anti-mouse IgG as secondary antibody at 1:8000 dilution. The immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Aylesbury, U.K.). Blots were then stripped, re-probed with an anti-β-actin (1:10000) and then with alkaline peroxidase-conjugated anti-mouse IgG (1:10000), developing in an identical manner for assessing β-actin protein levels to ensure even loading. All western blotting exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Quantity One software with a GS-800 densitometer (Bio-Rad).

**Immunofluorescent Analysis** For immunofluorescent analysis, cells were seeded onto sterile slide cover slips in 24 well plates with indicated treatments. They were then briefly washed three times with PBS, and fixed with 0.1% Triton X-100–4% paraformaldehyde for 30 min. Cover slips were blocked with 2% bovine serum albumin (BSA) in PBS and proved with anti-Cx43 1:1500 diluted in 2% BSA in PBS, the primary antibodies with a 3-h incubation at room temperature. Cells were washed, followed by the addition of FITC anti-mouse IgG at 1:400 dilution in 2% BSA in PBS for 1 h at room temperature. The nuclear staining was performed with Hoechst 33258 at 37 °C for 5 min. Cover slips were finally washed three times with PBS and mounted using 95% glycerol-PBS, samples were observed (×400) using an Olympus fluorescence microscope.

**RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the instructions of the manufacturer. Complementary DNA (cDNA) was synthesized from 1 μg RNA using the standard procedure with avian myeloblastosis virus reverse transcriptase (Promega) to generate 20 μl of cDNA at 42 °C for 60 min. For polymerase chain reaction (PCR) quantification, 2 μl of cDNA reaction was amplified in a 20 μl standard PCR reaction. PCR was initiated at 94 °C for 3 min followed by 30 cycles consisting of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C, with the final cycle extended to 10 min at 72 °C, followed by termination at 4 °C. The following primers were used: for mouse Cx43, forward primer 5'-TTGGCTCACGTTGTCTATGT-3' and reverse primer 5'-ACCTCTCATTTCACTTGCGT-3', product size 171 bps; for mouse β-actin, forward primer 5'-ACGGCCAGTCATCTACATTG-3' and reverse primer 5'-CTCTTCCTTCATGT-3', product size 351 bps. The detection of β-actin transcripts provided an internal control in PCR, stan-
dardizing the quantity of input cDNA. PCR products were analyzed on an ethidium bromide-stained 1.5% agarose gel. Bands were quantified by densitometric analysis. The ratio of Cx43 against β-actin expression was assessed and results were shown as electropherograms.

**Statistical Analysis of Data** The statistical analysis between groups was performed by unpaired Student’s t-test with Sigmaplot 10.0 software. Data were presented as mean±S.E.M. Differences with *p*<0.05 were considered significant.

**RESULTS**

**Cell Viability Measurement** In order to exclude the influence of cell viability on GJ function, we firstly investigated the effects of 2-APB and its analogues on cell viability by MTT assay. The results shown in Fig. 1 demonstrated that these agents had no significant effect on cell viability even at high concentrations [2-APB up to 50 μM, DPBA up to 30 μM, DPTTF, DPDM and PHT up to 100 μM (data not shown)]. The cell viability under any experimental conditions was more than 85%, and no alteration of either cell morphology or adhesion was exhibited (data not shown). These results prompted us to use 2-APB at concentrations comprised between 1 and 50 μM and DPBA between 1 and 30 μM in following experiments.

**Influence of 2-APB and Its Analogues on GJ Function**

GJ function was assessed by the “parachute” assay for dye coupling as described in Materials and Methods. Donor cells loaded with the nontransferable membrane dye DiI and the junction-permeable dye calcein were seeded onto unlabeled receiver cells. Experiments were carried out in the presence of 2-APB, its analogues and TPA, a widely used inhibitor of GJ composed of Cx43, respectively. The seeded donor cells were incubated with receiver cells for 4h to allow the dye to transfer through GJ channels. GJ function was assessed as the number of receiver cells containing calcein from a donor cell, normalized to that for control conditions (with DMSO treatment).

As shown in Fig. 2A, 2-APB (50 μM) as well as DPBA

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**Fig. 1. Effects of 2-APB and DPBA on Cell Viability**

Cells were treated with indicated concentrations of 2-APB or DPBA for 48h after being seeded into 96-well plates for 1d, and subsequently cell viability was determined, using MTT assay. The value of vehicle controls, treated with DMSO, was set at 1.0. Data represent the mean±S.E.M. of three-five independent experiments. *p*<0.05 vs. vehicle control.

**Fig. 2. Effects of 2-APB and Its Analogues on Dye Coupling through GJs Composed of Cx43 in TM3 Cells**

A, the dye spread of cells treated with 2-APB (50 μM), DPBA (30 μM), DPTTF (100 μM), DPDM (100 μM), PHT (100 μM) as well as TPA (50 nM) for 4h. B and C, the dye spread of cells treated with a range of 2-APB (B) and DPBA (C) concentrations for 4h. GJIC was assessed as the average number of receiver cells containing calcein from each donor cell, an average for each experiment of DMSO group was set at 1.0, the average of each other group was normalized to controls with DMSO treatment. Data represent the mean±S.E.M. of three-five independent experiments. *p*<0.05 vs. vehicle control.
(30 μM), substantially reduced the dye spread between the TM₄ Sertoli cells, whereas DPTTF, DPDM and PHT had essentially no effect on the dye coupling even at the concentration of 100 μM. TPA markedly inhibited the junctional coupling.

Figure 2B showed that the inhibitions of GJ by 2-APB and DPBA were in a concentration-dependent manner. The inhibitory effects of both compounds increased as the concentrations extended. Minimal inhibition occurred at 1 μM and maximum effect was achieved at 30 μM for 2-APB and 100 μM for DPBA, respectively. Notably, the inhibition of GJ induced by DPBA was more potent than that by 2-APB at the same concentrations (Figs. 2B, C). These results were in agreement with our previous findings which showed that both 2-APB and DPBA repressed GJ in cultured HeLa cells transfected with Cx32, and the inhibitory effect of DPBA was more potent than that of 2-APB.²⁶)

Effects of 2-APB and DPBA on GJ Are Reversible

Previous studies showed a rapid and reversible effect of 2-APB.³²,³³) In order to determine whether the inhibitory effects of 2-APB and DPBA on GJ were reversible, TM₄ cells were pretreated with 2-APB or DPBA at the highest nontoxic concentration (50, 30 μM, respectively) for 4 h and then the cells were incubated in drug-free medium for another 4 h. During the last 4 h, the donor cells were plated onto the receiver cell monolayer. The results illustrated in Fig. 3 indicated that dye coupling between cells was repressed when the two compounds were present. Whereas, GJ function recovered after the two agents were removed from external medium. These results suggested a totally reversible action of 2-APB or DPBA on GJ channels.

2-APB and DPBA Reduce Expression of Cx43 Protein

One possible mechanism by which GJ function might be suppressed is the down-regulation of Cx expression. We detected the amount of Cx43 using western blotting assay after 4 h treatment with or without the two tested agents. To concentrate the different Cx43 species in a single band, we used 15% polyacrylamide gel. As shown in Fig. 4A, treatment of the cells with 50 μM 2-APB and 30 μM DPBA for 4 h induced a markedly decrease in immunolabeling of Cx43. Meanwhile, DPTTF, DPDM and PHT did not affect expression of Cx43 (Fig. 4A), conforming to their inaction on GJ function.

**Fig. 3.** Reversible Inhibition of 2-APB and DPBA on GJs by Short Term Treatment

Cells were pre-incubated with 50 μM 2-APB (A) and 30 μM DPBA (B) for 4 h respectively before performing the dye transfer experiment, after which donor and receiver cells were co-cultured in the presence (agent) and absence (washout) of the agents for another 4 h, and subsequently GJ function was determined as described in Materials and Methods. Data represent the mean±S.E.M. of three independent experiments. *p<0.05 vs. vehicle control.

**Fig. 4.** Effects of 2-APB and Its Analogues on the Amount of Cx43 Protein in TM₄ Cells

A, western blotting analysis (15% polyacrylamide gel) was performed with total proteins extracted from cells with different treatments. B, the decrease in different Cx43 bands of cells treated with 2-APB (50 μM), DPBA (30 μM) and TPA (50 nM) for 4 h by western blotting analysis on an 8% polyacrylamide gel. P₀, non-phosphorylated Cx43; P₁ and P₂, phosphorylated forms of Cx43. Data obtained by densitometry represent the mean±S.E.M. of total Cx43/actin band densities of three determinations for each treatment condition. *p<0.05 vs. vehicle control.
By using more resolvent 8% polyacrylamide gel, which separates non-phosphorylated Cx43 (P0) and phosphorylated Cx43 (P1 and P2),34 we found that 2-APB and DPBA did not change the number of detected bands, but rather reduce all bands uniformly (Fig. 4B). The depressive amplitudes of the total amount of Cx43 by 2-APB and DPBA with 4 h treatment were approximately the same as that from the experiments using 15% polyacrylamide gel (Fig. 4A). These results indicated that 2-APB and DPBA decreased the level of Cx43 expression without affecting its phosphorylation state.

Figure 4B showed that exposure of TM4 cells to 50 nM TPA for 4 h resulted in a greater decrease in Cx43 level comparing to that of 2-APB or DPBA. Several lines of evidence demonstrated that TPA could inhibit GJ function in a number of cell types via reduction of Cx43 expression.35,36 The present results were consistent with these reports.

Effects of 2-APB and DPBA on the Number of GJs Composed of Cx43 Localized on Cell Membrane In order to assess whether the depressed GJ function was due to the alteration of the localization of GJ composed of Cx43 on cell membrane, immunofluorescent analysis was performed in TM4 cells. In control group (with DMSO treatment), numerous Cx43-specific punctuate spots were observed, predominantly along the plasma membrane at cell–cell contacts (Fig. 5), confirming the localization of Cx43 in intact cells. Consistent with the results of western blotting, a decrease in the number of GJs composed of Cx43 was demonstrated after treatment of the cells with either 2-APB or DPBA for 4 h (Fig. 5).

Effects of 2-APB and PDPA on the Level of Cx43 mRNA To further test whether the modulation of Cx43 expression by 2-APB and PDPA occurred at transcriptional level, we measured the expression of Cx43 mRNA by semi-quantitative RT-PCR. The results shown in Fig. 6 indicated that neither 2-APB nor PDPA, at 2 h or 4 h time point, elicited a noticeable change in the amplified PCR product for the Cx43 transcript in either sample (compare lanes 2—5 vs lane 1), suggesting that 2-APB and PDPA did not affect the level of Cx43 mRNA.

Effects of Lysosomal and Proteasomal Inhibitors on the 2-APB- or DPBA-Induced Decrease in Cx43 Expression The reduction of Cx43 protein and non-alteration of Cx43 mRNA induced by 2-APB and PDPA suggest that increased Cx degradation may be involved in the reduction of Cx43 protein. Degradation of Cx43 involves both the lysosome and the proteasome pathways.37,38 To delineate the degradation site of Cx43 induced by 2-APB and PDPA, cells were treated with the two agents and specific inhibitors of the lysosomal and proteasomal pathways for 4 h. Leupeptin (Leu), a potent lysosomal inhibitor, is widely used in the study of Cx43 degradation.39,40 Proteasomal proteolysis is inhibited by ALLN, which is highly specific for the proteasomal pathway.41 As shown in Fig. 7, inhibitors of both the lysosomal and the proteasomal pathways did not enhance the total amount of Cx43 within 4 h. However, Leu completely counteracted the decrease in Cx43 level induced by either 2-APB or DPBA, while ALLN partially prevented the reduction of Cx43 protein (Figs. 7A, B), indicating the induction in Cx43 degradation involved both lysosomal and proteasomal pathways.
DISCUSSION

We previously demonstrated that 2-APB and DPBA reduced the permeability of purified hemichannels and the junctional channels composed of Cx26/Cx32 by means of direct interaction with the Cxs. In the present study, the effects of these compounds on the function of GJ composed of Cx43, another important member of the Cx family, were explored. We found that 2-APB and DPBA could reversibly inhibit the function of Cx43 channel in a concentration-dependent manner in TM4 Sertoli cells, whereas other analogues of 2-APB, DPDM, DPTTF and PHT, had no effect on the GJ channels.

The permeability of GJ is controlled by both gating state and number of Cx channels on cell membrane. To explore the mechanisms of GJ inhibition, the effects of 2-APB and DPBA on the expression and distribution of Cx43 were investigated by western blotting and immunofluorescent analysis, respectively. Both the level of Cx43 protein and the number of GJs composed of Cx43, were reduced by treatment with 2-APB and DPBA for 4 h, consisting with their effects on dye coupling between cells. These results suggest the interruption of GJ function by 2-APB and DPBA may result from the decrease in Cx43 expression.

Theoretically, the reduction of Cx43 protein might be caused either by decreased production or enhanced breakdown of the protein. To evaluate these possibilities, the mRNA level of Cx43 was determined and degradation of Cx43 was assessed by using lysosomal and proteasomal inhibitors. The fact that the level of Cx43 mRNA at steady state being not changed by 2-APB or DPBA suggests that modification of Cx43 degradation may be responsible for the reduction of Cx43 protein level in the short term exposure. It has been demonstrated that both lysosomal and proteasomal pathways are involved in Cx degradation. Consistent with these studies, the lysosomal and proteasomal proteolytic systems are both shown to play an important role in the 2-APB- or DPBA-induced degradation of Cx43 in the present study. Leu completely counteracted the decrease in Cx43 level induced by either 2-APB or DPBA, while ALLN partially prevented the reduction of Cx43 protein, suggesting that the lysosomal pathway plays a dominant role in 2-APB- or DPBA-induced degradation of Cx43 in the present study. Leu completely counteracted the decrease in Cx43 level induced by either 2-APB or DPBA, while ALLN partially prevented the reduction of Cx43 protein, suggesting that the lysosomal pathway plays a dominant role in 2-APB- or DPBA-induced degradation of Cx43 in the TM4 cells. The present study did not observe the increase in Cx43 amount after treatment with these inhibitors, which were proved in other studies. We suppose the discrepancy with our results possibly due to differences in cell type or cell culture conditions, or a short treatment time, for that some reports demonstrated that treatment with Leu or ALLN for 5 h was not sufficient to increase intracellular Cx43 amount.

The mechanisms underlying the increase in Cx43 degradation induced by 2-APB and DPBA are unknown. It has been suggested that phosphorylation of Cx is related to its degradation. However, the phosphorylation state of Cx43 was not changed during 2-APB or DPBA treatment (Fig. 4B). Addition of ubiquitin has also been demonstrated to target some proteins for proteasomal degradation and other proteins to the lysosome. Cx43 is a ubiquitinated protein, and some of the degradation of Cx43 are ubiquitin-dependent. The study by Ohzono et al. indicated that the modulation of ubiquitination of Cx43 induced by Neddd4-interacting protein 2 (NDFIP2) were tightly linked Cx43 expression and that the polyprolinetyrosine (PY) motifs in the N-terminal of Cx43 played an important role in the process. Hence, ubiquitin-mediated degradation of Cx43 may be rendered as a possible mechanism responsible for the alteration of Cx43 expression induced by 2-APB and DPBA. Further studies are necessary to determine the involvement of ubiquitin in this process.

Cx43 is rapidly turned over in cells with the life time ranging from 1 to 4 h. Such dynamism of Cx43 implies that small changes in degradation of the protein may be critical for controlling the abundance of GJs and the extent of intercellular communication. Therefore theoretically, the de-

Figure 7. 2-APB- or DPBA-Induced Decrease in Cx43 Protein Level Involves an Effect on Cx43 Degradation

A, cells were treated with 2-APB (50 μM) alone or with either lysosomal inhibitor leupeptin (Leu; 25 μM) or proteasomal inhibitor ALLN (25 μM) for 4 h. B, cells were treated with DPBA (30 μM) alone or with either lysosomal inhibitor leupeptin (Leu; 25 μM) or proteasomal inhibitor ALLN (25 μM) for 4 h. Aliquots (20 μg) of total protein were separated by 8% SDS-PAGE and probed with a monoclonal anti-Cx43 antibody. Data represent the mean ± S.E.M. of three to four independent experiments. * p<0.05 vs. vehicle control; † p<0.05 vs. 2-APB alone (A) or DPBA alone (B).
increased GJ function due to the degradation of Cx43 induced by short term treatment with 2-APB or DPBA will be gradually recovered with Cx43 regeneration upon withdrawal of the agents. To assess the precise turnover of Cx43 protein levels, however, more complicated techniques, such as pulse labeling and chasing, are required.

At present we cannot exclude the possibility that 2-APB and DPBA might act directly on Cx43 channels, although we did not investigate their interaction further. In most reports, 2-APB and DPBA were shown to exert their inhibitory effects by directly blocking protein channels. For example, using reconstituted Cx hemichannels obtained from native tissues and stably-transfected cells, we characterized the direct inhibitions of Cx26 and/or Cx32 channels by 2-APB and DPBA. Some studies also implicated a reduction in junctional channel open probability induced by 2-APB through inhibition of electrical communication or through a direct extracellular action on Cx. Thus a comparative analysis among the tested compounds to find the pharmacophore for Cx43 channel inhibition is necessary.

From the structural features identified among active and inactive 2-APB analogues that are responsible for the activity (seen in Fig. 8 for structures), we found that the presence of the boron atom in 2-APB is absolutely required to inhibit Cx43 channels, for the other three analogues absent of the boron atom are lack of this inhibitory action. On the other hand, diphenylboronic moiety also seems to be required to exert the inhibitory activity, and the number of moiety is related to the potency of chemicals. For example, DPBA, containing two diphenylboronic groups, exhibits a stronger and longer inhibition than 2-APB does. However, attached a tertiary or tetrahedral carbon to the diphenyl groups will cancel the ability of DPDM, DPTTF or PHT to inhibit Cx43 channels (Fig. 8).

Moreover, it should be noteworthy that the ethanolamine chain of 2-APB can form an internal coordinate N→B bond with tetrahedral boron, which results in the formation of a boraoxazolidine ring (Fig. 8, 2-APB monomer ring). It is believed that such a five-member ring structure, unmodified, is an important component of the pharmacophore by which 2-APB inhibits calcium release from internal stores. DPDM cannot form such ring-structure, and PHT has a highly modified five-member ring, yet both of them are ineffective on Cx43 channels. Therefore, the structure–effect relationship is similar to those for inhibition of calcium release. Notably for PHT, the interpretation of lack inhibition needs to be caution. One is that extensive modification of the five-member ring cannot be tolerated. The other is, PHT, with two amines, does have a greater polar character. Due to its polar nature, it binds strongly to serum or membrane proteins, and this effect increases as pH is increased, so its ineffectiveness at blocking Cx43 channels in the cultures may be caused by binding to member proteins in the culture medium.

Together, the structural basis for these differences is unclear, but may be related to the absence of the diphenylboronic moiety in DPTTF, DPDM and PHT, or to the tendency of 2-APB (but not DPDM) to form a monomer ring without a highly modified five-member ring. When comparing the effects of 2-APB and its four analogues on GJ channels composed of different isotypes, we find that 2-APB and DPBA are the only two compounds that have nonspecific activities, although with different mechanisms. Thus 2-APB and DPBA seem to be common inhibitors of Cx26, Cx32 and Cx43 channels, suggesting that 2-APB might serve as template for the development of specific and effective antagonists of GJ channels. Further structure–activity studies are still required to explain their pharmacological differences clearly and to define the site(s) of action explicitly.

In conclusion, the present results for the first time demonstrate that 2-APB and DPBA reversibly inhibit the communication of GJ composed of Cx43 through reduction of Cx43 protein in TM4 Sertoli cells. This new finding may be helpful in extending the knowledge of this kind of compounds as a “blocker” in GJ research.

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REFERENCES
