ELSEVIER

European Journal of Pharmacology 442 (2002) 29–36

ejp

www.elsevier.com/locate/ejphar

Protein kinase C modulators enhance angiotensin II desensitization of guinea pig ileum via maxi-K^+ channels

Jeannine Aboulafia, Bagnólia A. Silva[1], Viviane L.A. Nouailhetas*

Department of Biophysics, Universidade Federal de São Paulo-Escola Paulista de Medicina, Rua Botucatu, 862, 7° andar, 04023-062 São Paulo, SP, Brazil

Received 21 January 2002; received in revised form 25 February 2002; accepted 1 March 2002

Abstract

We investigated the role of protein kinase C in the desensitization of the angiotensin II-induced contraction of guinea pig ileum. In contrast to their antagonistic effects on enzymatic activity, both activator and blockers accelerated the dissipation of the 10^{-7} M angiotensin II isometric contractile response. These agents indirectly activated maxi-K^+ channels in cell-attached membrane patches from freshly dispersed myocytes bathed in high-K^+ solution and clamped at -40 mV. In parallel with the contractile responses, fura 2-loaded myocytes bathed in Tyrode solution showed additive increases in $[Ca^{2+}]_i$ in response to both angiotensin II and phorbol dibutyrate (PDB). The PDB-promoted increase of the rate of angiotensin II desensitization was completely abolished by pretreatment of the tissue strips with 93 nM iberiotoxin or 8 mM KCl. Thus, we conclude that protein kinase C modulators promote faster angiotensin II desensitization by recruiting maxi-K^+ channels and inducing membrane repolarization rather than by affecting the protein kinase C activity.

Keywords: Angiotensin II desensitization; Phorbol dibutyrate (PDB); Membrane repolarization; Protein kinase C inhibitor; Maxi-K^+ channel recruitment; Ileum, guinea pig

1. Introduction

Angiotensin II, besides its potent vasoconstrictor action and other physiological effects (for a review, see Timmermans et al., 1993), has a direct action on the guinea pig ileum (Ohashi et al., 1967; Paiva et al., 1976) through its binding to the angiotensin AT_1 receptor subtype, which leads to the formation of inositol trisphosphate and diacylglycerol (Griendling et al., 1994). Its contractile response has two components: a fast and transient increase of the tonus, the phasic component, followed by a partial relaxation of the tissue to a sustained tonus, the tonic component. Prolonged treatment of this tissue with angiotensin II promotes a gradual decay of this component near to basal levels, usually within 15 min (Oshiro et al., 1989). This response, named desensitization, is mainly related to the state of electrical polarization of the plasma membrane, which is determined by the level of maxi-K^+ channels recruited by this agonist (Silva et al., 1999), and does not occur when the guinea pig ileum is stimulated with acetylcholine (Aboulafia et al., 1987) or KCl, since their tonic components remain.

The molecular mechanisms underlying desensitization of angiotensin AT_1 receptor-mediated responses in vascular smooth muscles appear to involve receptor phosphorylation, downregulation, and internalization (Wardle et al., 1997), or an additional protein kinase C-independent mechanism, as described for high agonist concentrations in Chinese hamster ovary cells (Tang et al., 1995). In guinea pig ileum, based on isotopic $^{45}Ca^{2+}$ uptake experiments with cultured cells of the longitudinal layer of this tissue, Shimuta et al. (1990) proposed that the angiotensin II desensitization of the contractile response may be due to a negative feedback mechanism mediated by protein kinase C affecting a step in the stimulus–response chain after phospholipase C activation. The usual pharmacological tools to study the participation of protein kinase C on a cellular mechanism are powerful inflammatory and tumor-promoting compounds which activate protein kinase C and are, thus, able to mimic responses mediated by the diacylglycerol pathway. As a representative of this class of compound, the phorbol ester 12, 13-dibutyrate (PDB) was also described as affecting maxi-K^+ channel activity in clonal rat anterior pituitary cells (Shipston and Armstrong, 1996). This effect, which would alter the electrical polarization state of the plasma membrane, renders

* Corresponding author. Tel.: +55-11-5572-4583; fax: +55-11-5571-5780.

E-mail address: vivi@biofis.epm.br (V.L.A. Nouailhetas).

[1] Present address: Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, Paraíba, PB, Brazil.

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01500-5

particularly interesting the study of this agent and protein kinase C inhibitors on the regulation of the contractile tonus of the guinea pig ileum, a potential-dependent phasic smooth muscle (Bolton, 1979), and on maxi-K^+ channel activity in freshly dispersed myocytes from this tissue, an experimental model where maxi-K^+ channels are well preserved in contrast to cultured cells, where this channel population is scarcely present (Nouailhetas et al., 1994).

In the present study, we reinvestigated the role of protein kinase C on the desensitization of the angiotensin II contractile response of the guinea pig ileum by using a protein kinase C activator (PDB) and inhibitors such as chelerythrine (Herbert et al., 1990), bisindolylmaleimide (Gekeler et al., 1996), or the indolocarbazole Gö 6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-a]pyrrolo[3,4-c]-carbazole) (Martiny-Baron et al., 1993). At a whole tissue level, the effect of these modulators was studied on the angiotensin II contractile response and on its susceptibility to changes in the membrane potential induced either by pretreating the tissue with a specific maxi-K^+ channel blocker, iberiotoxin (Candia et al., 1992), or by adding a KCl concentration which does not induce a contraction per se to the external medium (Silva et al., 1999). At a cellular level, we verified whether positive and negative modulators of protein kinase C activity are able to regulate maxi-K^+ channel currents in freshly dispersed intestinal smooth muscle cells. As far as we know, we have demonstrated a new interesting effect of these opposite protein kinase C modulators: both PDB and chelerythrine indirectly activate maxi-K^+ channel currents in cell-attached membrane patches of freshly dispersed cells of the longitudinal layer of the guinea pig ileum, most likely by increasing the intracellular Ca^{2+} concentration. In addition, we concluded that these agents promote a faster dissipation of the angiotensin II tonic component in the guinea pig ileum, not by regulating a protein kinase C negative feedback mechanism, as occurs in cultured cells (Shimuta et al., 1990), but probably by promoting additional recruitment of the maxi-K^+ channel population, thus leading to a faster repolarization of the membrane potential.

2. Materials and methods

2.1. Animals

Male or female albino guinea pigs, weighing between 200 and 250 g, were killed by decapitation.

2.2. Cell preparation

Guinea pig ileum smooth muscle cells were isolated according to the method described by Romero et al. (1998). Briefly, 2.5-cm segments of the longitudinal muscle layer of the guinea pig ileum were washed in 5 ml Ca^{2+}-free solution and exposed to Ca^{2+}-free solution containing 0.5 mg/ml collagenase, 0.3 mg/ml pronase (from *Streptomyces griseus*) and 2.0 mg/ml bovine serum albumin for 7 min at room temperature. The enzymatic digestion was interrupted by washing the tissue fragments with high Ca^{2+} solution containing 2.0 mg/ml bovine serum albumin and 0.1% trypsin inhibitor. The digested fragments were rinsed in Ca^{2+}-free solution and the cells were released by gently sucking the tissue fragments up and down in a blunt glass pipette. Cells were collected by centrifugation at $700 \times g$ for 30 s and the cell pellet was resuspended in Ca^{2+}-free solution, seeded on circular cover slips and kept at 4 °C for 1 h. At the time of the experiment, one cover slip was washed with the appropriate saline solution and transferred to the stage of a microscope for electrophysiological measurements.

2.3. Recording techniques

Single channel currents were recorded using either the inside–out or cell-attached modes of the patch-clamp technique (Hamill et al., 1981). Patch electrodes were made of borosilicate glass (Garner Glass, Claremont, CA, USA) by means of a two-stage puller (model PP-83, Narishige, Japan) and fire-polished (model MF-83 forge, Narishige) to a final pipette tip resistance of 5–10 MΩ. A 1 M KCl-agar bridge connecting the Ag–AgCl reference electrode was used to ground the bath solution. The cells and the electrode were visualized with an inverted microscope (model Diaphot, Nikon, Japan). Single channel currents were captured and amplified by means of a patch-clamp amplifier (EPC7, List Electronics, Darmstadt, FRG) and were stored on video tape (model PVC-6000, Philco-Hitachi, São Paulo, SP, BR) by means of an analog-to-digital converter (model DR-384, Neuro-Corder, Neuro Data Instruments, New York, NY, USA). Data were displayed on-line or from the video tape to a physiograph (model RS 3200, Gould, Cleveland, OH, USA) and to an oscilloscope (model MO 1221, Minipa, São Paulo, SP, BR), via a low-pass filter (eight-pole Bessel filter, Frequency Devices, Haverhill, MA, USA) at 3 kHz. All experiments were done at room temperature and at -40 mV membrane potential.

2.4. Data acquisition and analysis

Currents were acquired with a 16-bit analog-to-digital converter (TL-1 DMA Interface, Axon Instruments, Foster City, CA, USA) controlled by Fetchex software (pClamp 5.1, Axon). Records were analyzed using the computer program Transit (version 1.0, kindly offered by R. Latorre, Universidad de Chile). The duration and amplitude of each current were determined using idealized records from the original data, constructed through the recognition of the transitions between distinct levels. Transitions were detected when dI/dt (I = current amplitude) was higher than the slope threshold criterion, which was usually set at $\pm 3\sigma$ of the mean baseline noise. The open probability (P_o) corresponded to the fraction of time the channels spent at a given current level.

2.5. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements

Changes in $[Ca^{2+}]_i$ were monitored fluorometrically using the Ca^{2+}-sensitive probe {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′ -amino-5′ -methylphenoxy)-ethane-*N*,*N*,*N*′ ,*N*′ -tetracetic acid pentaacetoxymethyl ester}. This lipophilic acetoxymethyl ester, fura 2/AM, crosses the plasma membrane and is hydrolyzed by cytoplasmic esterases to yield the highly fluorescent, but impermeable form, fura 2. After an appropriate loading period at room temperature with 2 μM fura 2/AM and 2.5-ml suspensions of freshly dispersed myocytes from the longitudinal layer of the guinea pig ileum (10^6 cells/ml) in Tyrode solution, excess fura 2/AM was removed by washing and centrifugation at 715 × *g* for 2 min. The cell pellet was then resuspended in the same solution for the fluorescence studies. To determine $[Ca^{2+}]_i$, the fura 2 fluorescence was measured in 2.5-ml aliquots of cell suspensions (10^6 cells/ml) with a fluorophotometer SPEX AR-CM system (Spex Industries, Edisson, NJ, USA) with dual wavelength excitation (340 and 380 nm) and emission at 510 nm. The cell suspensions were maintained at 37 °C and continuously stirred. A calibration procedure was performed at the end of each experiment. Maximal fluorescence was obtained after treatment with 50 μM digitonin. Background fluorescence was determined by adding 5 mM Mn^{2+} to the suspension following the addition of digitonin. Minimal fluorescence (Ca^{2+}-free dye) was obtained by adding 10 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′ ,*N*′ -tetracetic acid (EGTA). The $[Ca^{2+}]_i$ was estimated by a ratiometric method (Grynkiewicz et al., 1985) from the fluorescence ratio (340/380 nm) according to the following equation, using the dissociation constant (K_d) of 224 nM:

$$[Ca^{2+}]_i = K_d \frac{[R - R_{min}](Sf_2)}{[R_{max} - R](Sb_2)}$$

where R is the fluorescence ratio (340/380 nm) at a given time, R_{max} is the maximal fluorescence ratio obtained after digitonin treatment, R_{min} is the minimum fluorescence ratio obtained in the presence of EGTA, and Sb_2 and Sf_2 are proportionality coefficients for fluorescence of Ca^{2+}-saturated fura 2 and free dye at 380 nm, respectively. The viability of the myocytes, measured by Trypan blue exclusion in a 10-μl aliquot of the cell suspension just before treatment with digitonin, was higher than 90%.

2.6. Tension measurements

Guinea pig ileum longitudinal smooth muscle strips were prepared as previously described (Paiva et al., 1988). Segments of longitudinal muscle (3–3.5 cm) were suspended in a 5-ml chamber containing Tyrode solution at 37 °C and bubbled with air. The isometric tension was recorded with a force transducer (Solução Integrada, São Paulo, BR, model TIM-05) connected to an amplifier (Solução Integrada, São Paulo, BR, model AECAD-0804). Acquisition and analysis of the isometric contraction were done with KitCad8 software (Soft and Solution, São Paulo, BR). Tissues were challenged with a maximal angiotensin II concentration for 15 min (control), washed, and after a 30-min resting period, to allow restoration of the initial contractile response (Paiva et al., 1976), the tissues were then submitted to the appropriate experimental condition. The desensitization rate was assessed from the slope of the linear portion of the tonic component, spanning the period from the beginning of tonus decay down to the initial point at which the final steady level was reached. The coefficient correlation values for each individual trace fitted by linear regression varied between 0.97 and 0.99.

2.7. Statistics

Desensitization rates are presented as means ± S.E.M., with n representing the number of experiments. Results were analyzed for statistical significance by Student's t-test. P values of < 0.05 were considered to represent statistically significant differences.

2.8. Solutions and drugs

The following solutions were used for the isolation of cells (in mM): Ca^{2+}-free solution: 132.4 NaCl, 5.9 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 11.5 glucose, 10 *N*-2-hydroxiethylpiperazine-*N*′ -2-ethanesulphonic acid (HEPES), pH 7.4 (NaOH 1 M), in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin; high-Ca^{2+} solution plus albumin: 2.0 mg/ml bovine serum albumin and 2.5 mM $CaCl_2 \cdot 2H_2O$ were added to Ca^{2+}-free solution. For patch-clamp experiments, the composition of both the bath and pipette solutions was (in mM): 150 KCl, 1 $MgCl_2.6H_2O$, 10 HEPES, pH 7.4 (KOH 1 M), referred as high-K^+ solution. In patch-clamp experiments, all chemicals were added to the cells by perfusion of homogeneous solutions, and data were obtained after 3 min, so that the complete replacement of the volume of the chamber was guaranteed. The composition of the Tyrode solution was (in mM): 137 NaCl, 2.68 KCl, 1.36 $CaCl_2 \cdot 2H_2O$, 0.49 $MgCl_2 \cdot 6H_2O$, 12 $NaHCO_3$, 0.36 NaH_2PO_4, 5.5 D-glucose.

2.9. Chemicals

All chemicals were analytical grade. Penicillin, streptomycin, HEPES, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′ ,*N*′ -tetracetic acid (EGTA), trypsin inhibitor, serum bovine albumin, fura 2/AM, digitonin, Trypan blue dye, phorbol 12, 13-dibutyrate (PDB), dimethylsulfoxide and iberiotoxin were purchased from Sigma (St. Louis, MO, USA). Chelerythrine was from Alomone Lab (Jerusalem, IL), collagenase I (217 U/ml) was from Worthington Biochemical (Freehold, NJ, USA), pronase was from Boehringer (Mannheim, FRG), and bisindolylmaleimide I

hydrochoride and Gö 6976 were from Calbiochem (La Jolla, CA, USA). Angiotensin II is routinely synthesized in our laboratory. Stock solutions (1 mg/ml) were prepared in water and kept at 0°C, and an appropriate dilution was made at the moment of the experiment. The water-insoluble protein kinase C modulators, PDB and Gö 6976, were dissolved in dimethylsulfoxide, the final concentration of which was less than 0.1% and did not interfere with the experimental data obtained with any of the methods used. Other salts and D-glucose were from Merck (Darmstadt, FRG).

3. Results

3.1. Effect of protein kinase C modulators on the contractile response to angiotensin II and KCl

It has been suggested that desensitization of the contractile response of the guinea pig ileum to angiotensin II is due to negative feedback exerted by protein kinase C at a step beyond angiotensin AT_1 receptor activation (Shimuta et al., 1990). This hypothesis was reexamined by investigating the relationship between protein kinase C activity, modulated by an enzymatic activator (PDB) and three blockers (chelerythrine, bisindolylmaleimide, and Gö 6976), and the angiotensin II contractile response of the guinea pig ileum longitudinal layer. The contractile response to a maximal concentration of angiotensin II (Fig. 1A) is characterized by a transient phasic contraction followed by desensitization of the tonic component as it gradually fades down to the basal tonus within 15 min. Fig. 1A also illustrates that addition of 10^{-7} M PDB to the tonic component induced by 10^{-7} M angiotensin II caused a 3.6-fold increase of the desensitization rate. After removal of PDB and a 30-min resting period, the angiotensin II tonus decay was partially reversed (Fig. 1A, Trace Rev.). Although discrete as compared to the PDB effect, 10^{-5} M chelerythrine, 10^{-6} M Gö 6976 (data not shown), or 10^{-6} M bisindolylmaleimide (Fig. 1B) also tended to increase the rate of angiotensin II desensitization.

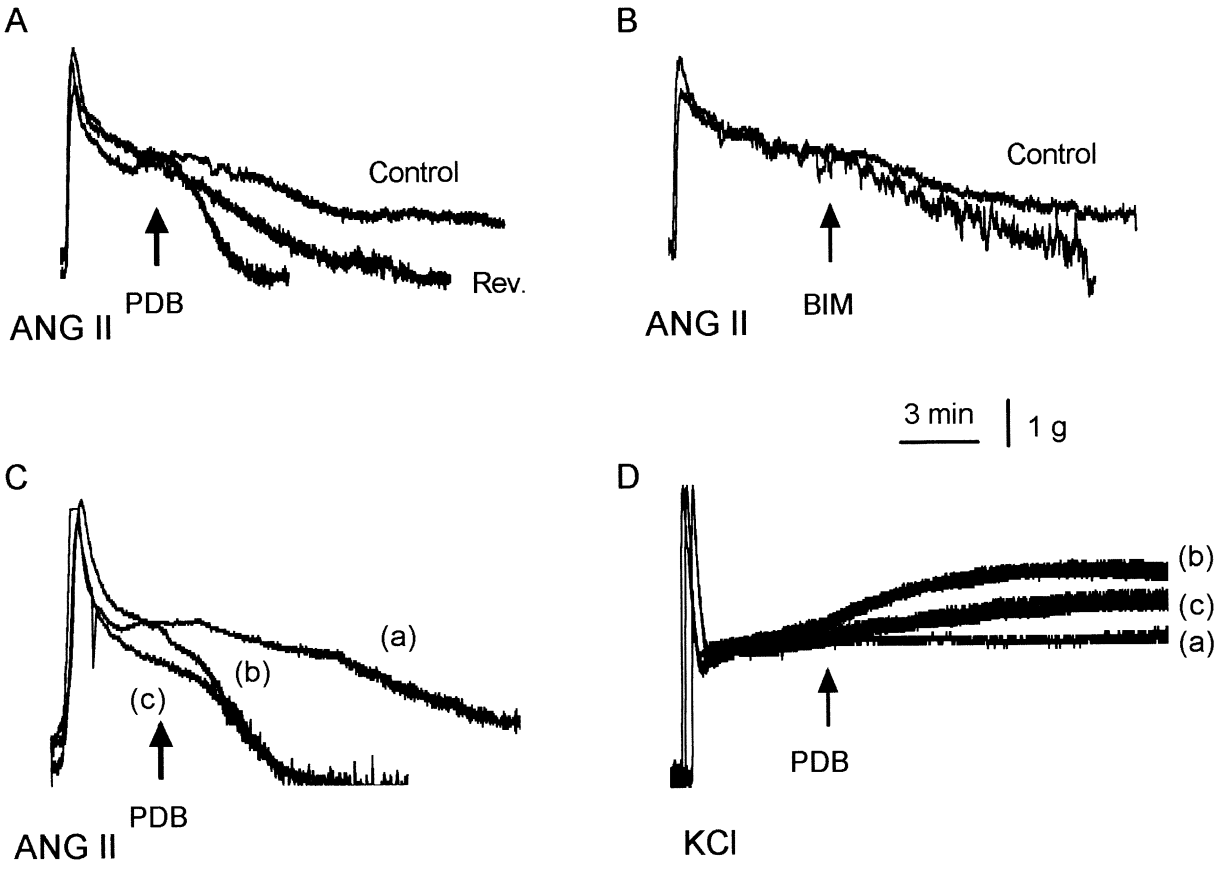

Fig. 1. Effect of protein kinase C modulators on the isometric tonic contractile responses of the guinea pig ileum induced by angiotensin II (ANG II) and KCl. (A) Effect of 10^{-7} M PDB on the tonic component induced by 10^{-7} M ANG II. The ANG II desensitization rate was calculated from the slope of the linear portion of the tonic component and was -0.11 ± 0.08 min^{-1} for the control condition and -0.39 ± 0.02 min^{-1} upon addition of PDB. *Trace Rev.*: partial reversibility of the ANG II contraction after the removal of PDB and a 30-min resting period ($n=8$). (B) Effect of 10^{-6} M bisindolylmaleimide (BIM) on the isometric tonic component induced by 10^{-7} M ANG II ($n=3$). (C, D) Control traces induced by the stimulant agents (a) and effect of 10^{-7} M PDB, in the absence (b) and presence (c) of 10^{-5} M chelerythrine, on the tonic component induced by 10^{-7} M ANG II (panel C) ($n=8$), and 40 mM KCl (panel D) ($n=3$). Chelerythrine was preincubated for 5 min before the stimulant challenges. Tissues were bathed in Tyrode solution at 37°C.

For PDB concentrations lower than 10^{-7} M, the effect on the tonic component was quite variable: while some preparations showed a consistent concentration-dependent relationship over the range 10^{-9} to 10^{-7} M, others were rather insensitive to PDB concentrations up to 3×10^{-8} M. Above this PDB concentration, the desensitization rate was not significantly different from that obtained with 10^{-7} M PDB. Thus, we used in the following experiments this maximal PDB concentration.

In order to study the effects of these protein kinase C-acting drugs on the initial phasic component of the contractile response, the tissue was preincubated with either 10^{-7} M PDB, 10^{-5} M chelerythrine, or 10^{-6} M bisindolylmaleimide for 5 min before the 10^{-7} M angiotensin II challenge (data not shown). No alterations were observed in the phasic component, but the tonic response dissipated even faster, following the same pattern described when the agent was added to the angiotensin II tonic component. This suggested that the enhanced dissipation of the tonic component induced by these agents did not depend on whether protein kinase C was already activated by angiotensin II.

In addition to the lack of antagonism between the effects induced by PDB and the protein kinase C inhibitors on the angiotensin II tonic contractile response, previous inhibition of protein kinase C activity by chelerythrine, bisindolylmaleimide, or Gö 6976 added 5 min before the administration of angiotensin II also failed to prevent or impair the PDB-induced increased rate of desensitization of the tonic component of the response to angiotensin II, as illustrated in Fig. 1C for the response induced in the presence of chelerythrine. This, however, was not observed when these agents were tested on the KCl-induced contraction, since addition of PDB caused a slight increase of the tonic component, which was partly reversed by previous inhibition of protein kinase C activity by chelerythrine (Fig. 1D).

3.2. Effect of PDB and chelerythrine on maxi-K^+ channel activity, and on $[Ca^{2+}]_i$

Since we have recently shown that modulation of maxi-K^+ channel activity is involved in the mechanism of the desensitization to angiotensin II of intestinal smooth muscle (Silva et al., 1999), the effect of PDB and chelerythrine was also investigated on this channel activity, using the cell-attached configuration of the patch-clamp technique (Hamill et al., 1981). At a membrane potential of -40 mV and with the intestinal myocytes bathed in high-K^+ solution, addition of 10^{-7} M PDB or 10^{-5} M chelerythrine promoted a reversible 100-fold and 66-fold increase of the channel activity, respectively, as illustrated in Fig. 2 for two different membrane patches where at least three maxi-K^+ channels were simultaneously open. However, addition of these two agents at the cellular face of inside–out membrane patches did not promote any alteration of the maxi-K^+ channel activity (data not shown), indicating that both PDB and chelerythrine require an intracellular messenger to activate the maxi-K^+ channel. Moreover, the enhanced maxi-K^+ channel activity induced by angiotensin II (Romero et al., 1998; Silva et al., 1999) was not altered by any further addition of PDB or chelerythrine to the bath solution (data not shown), suggesting that angiotensin II and these two protein kinase C modulators may share a common step in the transduction signaling pathway, and/or that the maxi-K^+ channels were already maximally activated by angiotensin II.

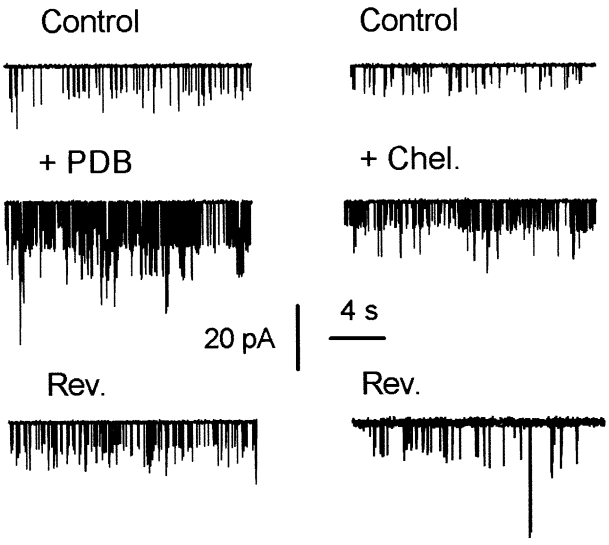

Fig. 2. Indirect effect of protein kinase C modulators on the maxi-K^+-channel activity of intestinal myocytes. K^+-currents were recorded from two distinct cell-attached membrane patches of freshly dispersed myocytes from the longitudinal layer of guinea pig ileum in the presence of high-K^+ solution in the bath and pipette, before (control), after addition of 10^{-7} M PDB or 10^{-5} M chelerythrine (Chel.), and 30 min after their removal from the bath solution (Rev.). Patches had a membrane potential of -40 mV. P_o values were 0.028, 0.310, and 0.034 in the absence of PDB, in the presence, and after its removal, respectively. In the case of chelerythrine, these values were 0.026, 0.167, and 0.051. Recording total time 100 ms, data sampling 1.25 kHz. Experiments at room temperature. Records are representative of four experiments.

Considering that in angiotensin II-treated myocytes, the sustained activation of the maxi-K^+ channel population has been attributed to an increased cytosolic Ca^{2+} concentration (Romero et al., 1998), we measured the free $[Ca^{2+}]_i$ in fura 2-loaded freshly dispersed myocytes from the longitudinal muscle of guinea pig ileum subsequently stimulated with angiotensin II and PDB, or vice versa. Fig. 3 shows that in cells bathed in Tyrode solution and stimulated with angiotensin II there was a fast and transient increase of the $[Ca^{2+}]_i$, attaining a stable level, which was followed by a further steady increase upon PDB challenge. When PDB was added first, there was a minor steady increase of the $[Ca^{2+}]_i$, which was promptly enhanced in response to the subsequent administration of angiotensin II. It is worth noting that regardless of the order of agent addition, the same final $[Ca^{2+}]_i$ was observed.

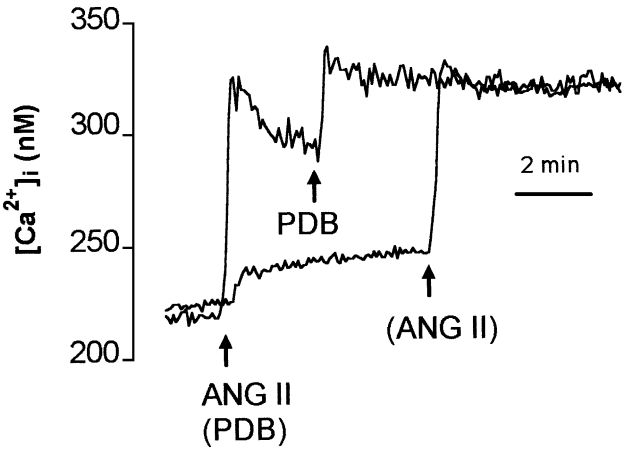

Fig. 3. Increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by subsequent treatments with 10^{-7} M PDB and 10^{-7} M ANG II, and vice versa, of fura 2-loaded intestinal myocytes from the longitudinal layer of the guinea pig ileum. The cells were bathed in Tyrode solution. The first stimulant agent, PDB or ANG II, was added to the external medium after a 1.5-min recording of the basal free Ca^{2+} level. Only cells with a stable basal level were used for the experiments. The second challenge was done at the indicated time. Records are representative of three experiments.

3.3. Effect of membrane depolarization on the rate of angiotensin II desensitization in the presence of PDB

To verify that the PDB-promoted increase of the rate of angiotensin II desensitization is also modulated by membrane repolarization, we studied the contractile response of angiotensin II-PDB-treated tissues when the membrane potential was altered, by either blocking maxi-K^+ channel activity or adding KCl to the physiological solution. Fig. 4 shows that preincubation for 5 min of the tissue strip with either 93 nM iberiotoxin, a well-known specific blocker of maxi-K^+ channel activity (Candia et al., 1992), or 8 mM KCl completely counteracted the PDB-increased rate of desensitization of the angiotensin II tonic contraction, since a sustained tonic component was observed under these experimental conditions.

4. Discussion

In this study, we provide evidence that protein kinase C is not responsible for the desensitization of the contractile response to angiotensin II in intestinal smooth muscle. This conclusion is based on the observation that the protein kinase C activator and inhibitors tested did not elicit opposite effects on the contractile response induced by angiotensin II and on maxi-K^+ channel activity. These agents accelerated the dissipation of the tonic contractile response and also positively modulated the activity of this channel population, probably by increasing $[Ca^{2+}]_i$. We, thus, propose that the faster desensitization of the angiotensin II tonic contraction promoted by these agents reflects their ability to induce a higher level of membrane repolarization by recruiting maxi-K^+ channels rather than their ability to modulate protein kinase C activity.

Despite the great number of studies regarding the role exerted by protein kinase C on the regulation of smooth muscle contractile function, this still remains unresolved because the results are often conflicting. These disparities are usually explained by differences in the tissues or animal species studied, such as varieties of protein kinase C isozyme expression, or are attributed to the distinct pharmacological tools used, such as protein kinase C activators or inhibitors, which have only relative specificity for protein kinase C as compared to other kinases, thus limiting the interpretation of the data in a physiological context (Singer, 1996). Conflicting results have been reported, but an extensive review of these would be beyond the scope of this study. Nevertheless, whatever the functional role of protein kinase C in guinea pig ileum is, our rationale to assess whether the desensitization of the contractile response induced by angiotensin II is a protein kinase C-dependent mechanism is that PDB, which is a protein kinase C activator, and chelerythrine, or any other protein kinase C inhibitor, must cause qualitatively opposite effects on the tonic contraction. Our results show that this was the case for the KCl-, but not for the angiotensin II-induced contractions. Indeed, while the KCl-induced tonic contrac-

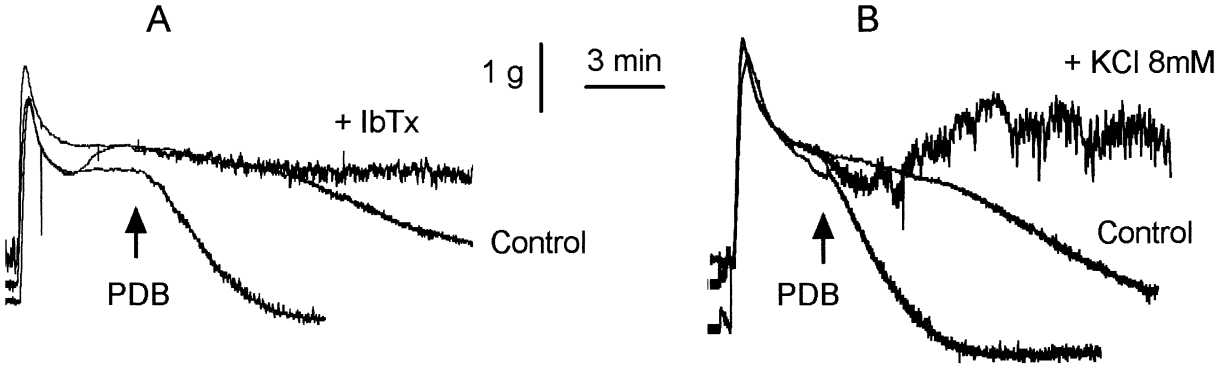

Fig. 4. Effect of (A) 93 nM iberiotoxin (IbTx) and (B) a membrane depolarization level achieved by 8 mM KCl on the ANG II tonic component in the absence (control) and presence of 10^{-7} M PDB added at the indicated time. Longitudinal guinea pig ileum muscle strips were incubated for 5 min with IbTx or KCl before the 10^{-7} M ANG II challenge. Tissues were bathed in Tyrode solution at 37 °C. Experiments with IbTx and KCl were carried out with different muscles strips, the superimposed records being representative of five experiments.

tions were slightly, but consistently, modulated by PDB and chelerythrine, the former enhancing the tonus and the latter partly reversing it (Fig. 1D), desensitization to angiotensin II occurred in the presence of either PDB (Fig. 1A), bisindolylmaleimide (Fig. 1B), chelerythrine, or Gö 6976 (not shown). PDB markedly accelerated the rate of desensitization to angiotensin II (Fig. 1A and Shimuta et al., 1990), while the protein kinase C inhibitors did not prevent or even delayed this process (Fig. 1C), having a slight and reproducible tendency to also dissipate the tonic component (Fig. 1B).

As these results strongly argue against a direct effect of protein kinase C on the angiotensin II desensitization, we raised the possibility that these agents were modulating the desensitization rate by promoting a higher level of membrane repolarization. This could be achieved by opening any K^+-channel population, which would repolarize the membrane by closing any depolarized activated membrane conductance, thus leading to the relaxation of this potential-dependent smooth muscle. As recently a relevant role was assigned to maxi-K^+ channels in the modulation of the angiotensin II desensitization of the guinea pig ileum (Silva et al., 1999), the effect of PDB and chelerythrine was studied on maxi-K^+ channel activity. In cell-attached experiments, both PDB and chelerythrine enhanced maxi-K^+ single channel currents (Fig. 2), which, in accordance with our hypothesis, would then shift the membrane potential toward a more repolarized state of the membrane. However, with the inside–out configuration, the two protein kinase C modulators did not alter maxi-K^+ channel activity when added to the cellular face of the patch (data not shown), indicating that there are no interaction sites for these agents at this side of the membrane, so that intracellular signaling is required to activate this channel population. Given that in angiotensin II-treated myocytes the sustained activation of the maxi-K^+ channel has already been attributed to an increased cytosolic Ca^{2+} concentration (Romero et al., 1998), that PDB increases intracellular Ca^{2+} (Rembold and Murphy, 1988), and that chelerythrine potentiates agonist-mediated Ca^{2+} influx (Shah et al., 1996), Ca^{2+} appears a plausible candidate. The results of the fura 2 experiments paralleled the isometric contractile response patterns obtained when the tissues were subsequently stimulated with PDB and angiotensin II, or vice versa. In fura 2-loaded guinea pig ileum myocytes, addition of PDB promoted a slight, but sustained, increase of the $[Ca^{2+}]_i$ (Fig. 3), probably to a level high enough to guarantee maxi-K^+ channel activation (Fig. 2), whereas the further sharp and sustained increase of the intracellular Ca^{2+} concentration induced by angiotensin II (Fig. 3) appeared to be sufficient to promote both the biphasic isometric response to angiotensin II and further activation of maxi-K^+ channels, leading ultimately to a faster rate of desensitization (Fig. 1A). So far, one must assume that PDB and angiotensin II have an additive effect on maxi-K^+ channel recruitment in the whole tissue, but not at a single channel level (data not shown). This is probably due to maximal activation of these channels by angiotensin II (Romero et al., 1998) because the membrane is chemically clamped at zero, because the cells are continually bathed in high-K^+ solution, thus disfavoring any further electrical effect on the voltage-dependent channels. Furthermore, the experimental conditions presented in Fig. 4 corroborate that the angiotensin II desensitization phenomenon is mainly controlled by maxi-K^+ channels, and not by any mechanism involving the modulation of protein kinase C activity. Actually, despite the presence of PDB, the dissipation of the angiotensin II tonic component was no longer observed when the contribution of maxi-K^+ channels was blocked by iberiotoxin (Fig. 4A), or by promoting an additional depolarization mechanism by adding 8 mM KCl to the medium (Fig. 4B).

Finally, two important differences must be pointed out between our experimental protocols and those described by Shimuta et al. (1990). First, the protein kinase C negative modulation, proposed by Shimuta et al. to explain the angiotensin II desensitization mechanism, was based on studies performed with cultured cells of the guinea pig ileum where maxi-K^+ channels are scarcely present (Nouailhetas et al., 1994), in contrast to the wide distribution of this channel population in the whole tissue as well as in the membranes of freshly dispersed cells (Romero et al., 1998). Second, their analysis relied on the effect of protein kinase C modulators on the $^{45}Ca^{2+}$ uptake induced by angiotensin II, which might be only one step in the agonist–response cascade, and not on the final response, i.e. the contractile response studied here. Therefore, at first sight, it seems that the comparison does not stand up. However, in the presence of iberiotoxin, a condition that simulates the cultured cells with regard to maxi-K^+ channel distribution, the increased rate of dissipation of the angiotensin II tonic component induced by PDB was completely blocked (Fig. 4). We infer that if protein kinase C plays a role in any step of the angiotensin II signaling cascade leading to desensitization, this will certainly be quenched in the presence of maxi-K^+ channels. Thus, cultured cells are an inappropriate model for studying the desensitization to angiotensin II of isolated tissue because these cells apparently lack an important regulator of relaxation of the guinea pig ileum.

In summary, the enhanced rate of desensitization of the tonic component of the angiotensin II response induced by PDB or protein kinase C blockers in the guinea pig ileum is a consequence of their ability to induce maxi-K^+ channel activation rather than their ability to modulate protein kinase C activity.

Acknowledgements

We are grateful to Dr. Maria Etsuko M. Oshiro and Dr. Alice T. Ferreira for support in the intracellular Ca^{2+} measurements, and to Nelson A. Mora, Chandler Tahan, and Andréa Simonato for their technical assistance. B.A. Silva was on a fellowship from Coordenação de Aperfeiçoamento

de Pessoal de Nível Superior. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Estado de São Paulo.

References

Aboulafia, J., Capocci, H., Paiva, A.C.M., Paiva, T.B., 1987. Sodium-dependence of the nonspecific desensitization of the guinea-pig ileum induced by acetylcholine and histamine. Br. J. Pharmacol. 90, 347–353.

Bolton, T.B., 1979. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59, 606–718.

Candia, S., Garcia, M.L., Latorre, L., 1992. Mode of action of iberiotoxin, a potent blocker of the large conductance Ca^{2+}-activated K^+ channel. Biophys. J. 63, 583–590.

Gekeler, V., Boer, R., Uberall, F., Ise, W., Schubert, C., Utz, I., Hofmann, J., Sanders, K.H., Schachtele, C., Klemm, K., Grunicke, H., 1996. Effects of the selective bisindolylmaleimide protein kinase C inhibitor GF 109203X on P-glycoprotein-mediated multidrug resistance. Br. J. Cancer 74, 897–905.

Griendling, K.K., Lassègue, B., Murphy, T.J., Alexander, R.W., 1994. Angiotensin II receptor pharmacology. Adv. Pharmacol. 28, 269–306.

Grynkiewicz, J., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 160, 3440–3450.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch. 391, 85–100.

Herbert, J.N., Augerau, J.M., Gleye, J., Manffrand, J.P., 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 172, 993–999.

Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, H., Hug, H., Marme, D., Schachtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. 268, 9194–9197.

Nouailhetas, V.L.A., Aboulafia, J., Frediani-Neto, E., Ferreira, A., Paiva, A.C.M., 1994. A Na^+-sensitive cation channel modulated by angiotensin II in cultured intestinal myocytes. Am. J. Physiol. 266, C1538–C1543.

Ohashi, H., Nomura, Y., Ohga, S., 1967. Effects of angiotensin, bradykinin and oxytocin on electrical and mechanical activities in the taenia coli of guinea-pig. Jpn. J. Pharmacol. 17, 247–257.

Oshiro, M.E.M., Shimuta, S.I., Paiva, T.B., Paiva, A.C.M., 1989. Evidence for a regulatory site in the angiotensin II receptor of smooth muscle. Eur. J. Pharmacol. 166, 411–418.

Paiva, T.B., Mendes, G.B., Aboulafia, J., Paiva, A.C.M., 1976. Evidence against cholinergic mediation of the effect of angiotensin II on the guinea-pig ileum. Pfluegers Arch. 365, 129–133.

Paiva, T.B., Paiva, A.C.M., Shimuta, S.I., 1988. Role of sodium ions in angiotensin tachyphylaxis in the guinea pig ileum and taenia coli. Naunyn-Schmiedberg's Arch. Pharmacol. 337, 656–660.

Rembold, C.M., Murphy, R.A., 1988. [Ca^{2+}]-dependent myosin phosphorylation in phorbol diester stimulated smooth muscle contraction. Am. J. Physiol. 255, C719–C723.

Romero, F., Silva, B.A., Nouailhetas, V.L.A., Aboulafia, J., 1998. Activation of Ca^{2+}-activated K^+ (maxi-K^+) channel by angiotensin II in myocytes of the guinea pig ileum. Am. J. Physiol. 274, C983–C991.

Shah, B.H., Shamim, G., Khan, S., Saeed, S.A., 1996. Protein kinase C inhibitor, chelerythrine, potentiates the adrenaline-mediate aggregation of human platelets through calcium influx. Biol. Mol. Biol. Int. 38, 1135–1141.

Shimuta, S.I., Kanashiro, C.A., Oshiro, M.E.M., Paiva, T.B., Paiva, A.C.M., 1990. Angiotensin II desensitization and Ca^{2+} and Na^+ fluxes in cultured intestinal smooth muscle cells. J. Pharmacol. Exp. Ther. 353, 1215–1221.

Shipston, M.J., Armstrong, D.L., 1996. Activation of protein kinase C inhibits calcium-activated potassium channels in rat pituitary tumour cells. J. Physiol. (London) 493, 665–672.

Silva, B.A., Nouailhetas, V.L.A., Aboulafia, J., 1999. Desensitization to ANG II in guinea pig ileum depends on membrane repolarization: role of maxi-K^+ channel. Am. J. Physiol. 277, C739–C745.

Singer, H.A., 1996. Protein kinase C. In: Bárány, M. (Ed.), Biochemistry of Smooth Muscle Contraction. Academic Press, New York, NY, pp. 155–165.

Tang, H., Shirai, H., Inagami, T., 1995. Inhibition of protein kinase C prevents rapid desensitization of type 1B angiotensin II receptor. Circ. Res. 77, 239–248.

Timmermans, P.B.M.W.M., Wong, G.L., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A.M., Smith, R.D., 1993. Angiotensin II receptor and angiotensin II receptor antagonists. Pharmacol. Rev. 45, 205–251.

Wardle, R.L., Strauss, J.D., Rembold, C.M., Murphy, R.A., 1997. Heterologous desensitization of smooth muscle to K^+ depolarization: retarded stimulus-$[Ca^{2+}]_i$ coupling. Am. J. Physiol. 272, C1810–C1820.