Activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (maxi-K\textsuperscript{+}) channel by angiotensin II in myocytes of the guinea pig ileum

FERNANDO ROMERO, BAGNÓLIA A. SILVA, VIVIANE L. A. NOUAILHETAS, AND JEANNINE ABOULAFIA
Department of Biophysics, Universidade Federal de São Paulo, Escola Paulista de Medicina, 04023-062 São Paulo SP, Brazil

Romero, Fernando, Bagnólia A. Silva, Viviane L. A. Nouailhetas, and Jeannine Aboulafia. Activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (maxi-K\textsuperscript{+}) channel by angiotensin II in myocytes of the guinea pig ileum. Am. J. Physiol. 274 (Cell Physiol. 43): C983–C991, 1998.—We investigated the regulation of the Ca\textsuperscript{2+}-activated K\textsuperscript{+} (maxi-K\textsuperscript{+}) channel by angiotensin II (ANG II) and its synthetic analog, [Lys\textsuperscript{2}]ANG II, in freshly dispersed intestinal myocytes. We identified maxi-K\textsuperscript{+} channel population in the inside-out patch configuration on the basis of its conductance (257 ± 4 pS in symmetrical 150 mM KCl solution), voltage and Ca\textsuperscript{2+} dependence of channel opening, low Na\textsuperscript{+}-to-K\textsuperscript{+} and Cl\textsuperscript{−}-to-K\textsuperscript{+} permeability ratios, and blockade by external Cs\textsuperscript{+} and tetraethylammonium chloride. ANG II and [Lys\textsuperscript{2}]ANG II caused an indirect, reversible, Ca\textsuperscript{2+}- and dose-dependent activation of maxi-K\textsuperscript{+} channels in cell-attached experiments when cells were bathed in high-K\textsuperscript{+} solution. This effect was reversibly blocked by DUP-753, being that it is mediated by the AT\textsubscript{1} receptor. Evidences that activation of the maxi-K\textsuperscript{+} channel by ANG II requires a rise in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) as an intermediate step were the shift of the open probability of the channel-membrane potential relationship to less positive membrane potentials and the sustained increase in [Ca\textsuperscript{2+}]\textsubscript{i} in [Ca\textsuperscript{2+}\textsuperscript{++}] in fura 2-loaded myocytes. The preservation of the pharmacomechanical coupling of ANG II in these cells provides a good model for the study of transmembrane signaling responses to ANG II and analogs in this tissue.

Longitudinal layer; patch clamp; intracellular calcium concentration; fura 2

ANGIOTENSIN II (ANG II) is an octapeptide hormone with a broad physiological spectrum. Besides its powerful vasoconstrictor action, this peptide regulates the cardiovascular, renal, neuroendocrine, and central nervous systems and stimulates protein synthesis in vascular smooth muscle cells and cell mitogenesis (for a review, see Ref. 26).

Two distinct isoforms of the ANG II receptor have been identified on the basis of their affinity to nonpeptide antagonists. The AT\textsubscript{2} receptor, which is blocked by PD-123177, is mainly present in fetal tissues and is less frequently observed in adult adrenal medulla, uterus, or brain (8, 26). It plays an important role in cellular growth (26). The AT\textsubscript{1} receptor subtype, which is blocked by DUP-753 (26), is distributed mainly in visceral smooth muscles, such as uterus and urinary bladder, and other tissues, such as adrenal gland, kidney, and liver (7). The AT\textsubscript{1} receptor has been cloned from vascular and adrenal cells and is a member of the guanine nucleotide protein-coupled (G protein) superfamily of receptors. These receptors are characterized by the seven transmembrane α-helix segments, and they mediate the ANG II effects on smooth muscle contraction, aldosterone secretion, and norepinephrine potentiation (26).

Activation of the AT\textsubscript{1} receptor mobilizes four distinct enzyme cascades [adenylate cyclase and phospholipases A\textsubscript{2}, C, and D (8)] through a nonidentified G protein. The most-studied signal transduction by ANG II is its binding to the AT\textsubscript{1} receptor, leading to the formation of two second messengers, inositol trisphosphate ([IP\textsubscript{3}] and diacylglycerol (DAG). The formed IP\textsubscript{3} in the sarcoplasm causes an increase of the cytosolic Ca\textsuperscript{2+} concentration due to the opening of IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} channels from the sarcoplasmic reticulum, thus triggering contraction. In addition, DAG activates protein kinase C, which may in turn stimulate protein phosphorylation and ionic channel activation (cf. Ref. 28). In addition, ANG II may also induce an increase of Ca\textsuperscript{2+} currents, blockade of K\textsuperscript{+} channels, and depolarization of the cell (cf. Ref. 28).

In the guinea pig ileum, ANG II depolarizes (24) the cells by activating nonspecific cation channels (21) and/or voltage-gated Ca\textsuperscript{2+}-channels, leading to enhanced Ca\textsuperscript{2+} and Na\textsuperscript{+} influxes (25) and increasing intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}). Depolarization and elevation of [Ca\textsuperscript{2+}]\textsubscript{i} would favor activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels (maxi-K\textsuperscript{+} channels). These channels, also referred as “intracellular Ca\textsuperscript{2+} indicators,” have been described in many smooth muscles (for a review, see Ref. 27), and they undergo positive or negative regulation by various stimulants, including ANG II (1), endothelin (11), substance P (19), and acetylcholine (4, 17). Although macroscopic K\textsuperscript{+} currents through maxi-K\textsuperscript{+} channels in intestinal myocytes of the guinea pig ileum have been already reported (31), their biophysical characteristics and modulation by distinct agonists, including ANG II, are not well established at the single-channel level yet.

In the present study, we used first the inside-out configuration of the patch-clamp technique (10) to identify the presence of maxi-K\textsuperscript{+} channels in the plasma membrane of freshly dispersed myocytes isolated from the longitudinal layer of the guinea pig ileum by determining some of their biophysical properties, like single-channel conductance, selectivity, Ca\textsuperscript{2+} and voltage sensitivity of the gating mechanism, and blockade by tetraethylammonium chloride (TEA\textsuperscript{+}) and Cs\textsuperscript{+}. Second, we used the cell-attached configuration to explore the modulatory role of ANG II and its synthetic analog, [Lys\textsuperscript{2}]ANG II, on the activity of these channels. Basically, we report the presence of maxi-K\textsuperscript{+} channels in
myocytes of the guinea pig ileum with properties very similar to those described in other tissues and the indirect and positive modulation of maxi-K\textsuperscript{+} channels by ANG II and [Lys\textsuperscript{2}]ANG II due to the increase in [Ca\textsuperscript{2+}], promoted by these peptides.

**MATERIALS AND METHODS**

Animals. Either male or female albino guinea pigs, weighing between 200 and 250 g, were used in this study.

Cell preparation. Smooth muscle cells of the guinea pig ileum were isolated according to the method described by Inoue et al. (14), with some modifications. The longitudinal muscle layer was peeled off and cut into 2.5-cm fragments. These tissues were washed in 5 ml Ca\textsuperscript{2+}-free solution and exposed to Ca\textsuperscript{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 twice in high-Ca\textsuperscript{2+} solution containing 2.0 mg/ml bovine serum albumin and 0.1% trypsin inhibitor. The digested fragments were rinsed six times in Ca\textsuperscript{2+}-free solution, and the cells were released by successively drowning the tissue fragments in and out of a blunt glass pipette. The cells were collected by centrifugation at 700 g for 30 s, and the cell pellet was resuspended in Ca\textsuperscript{2+}-free solution, seeded on circular coverslips, and kept at 4°C for 1 h. At the time of the experiment, one coverslip was washed with the appropriate saline solution and transferred to the stage of a microscope for electrophysiological measurements.

The freshly dispersed longitudinal smooth muscle cells of the guinea pig ileum had an elongated shape, 2–5 μm diameter and 150 μm length, with a central nucleus and a very clean plasma membrane. The viability of the cells was verified through the trypan blue exclusion test, and only cells completely relaxed were used for electrophysiological measurements. The yield of the process was quite variable, and some adjustments had to be done whenever a different enzyme lot was used.

Solutions and drugs. The following solutions were used for isolation of the cells (in mM): Ca\textsuperscript{2+}-free solution 132.4 NaCl, 5.9 KCl, 3.2 MgCl\textsubscript{2}, 1.0 CaCl\textsubscript{2}, 11.5 glucose, 10 N\textsubscript{2}-hydroxyethylpiperazine-N\textsubscript{2}-ethanesulfonic acid (HEPES), pH 7.4 (with 1 M NaOH), in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin and high-Ca\textsuperscript{2+} solution plus albumin (2.0 mg/ml bovine serum albumin and 2.5 mM CaCl\textsubscript{2} · 2H\textsubscript{2}O were added to Ca\textsuperscript{2+}-free solution). For inside-out patch experiments, the composition of both the bath and pipette solutions was (in mM) 150 KCl, 1 MgCl\textsubscript{2}, 6H\textsubscript{2}O, and 10 HEPES (pH 7.4, with 1 M KOH); these solutions are referred to throughout this study as high-K\textsuperscript{+} solution. For selectivity experiments, the KCl concentration of the high-K\textsuperscript{+} solution was replaced by 30 mM KCl or 120 mM NaCl plus 30 mM KCl; the other components remaining unchanged. In excised inside-out patch experiments, pipette and bath solutions were also referred to as external and internal solutions, respectively. Ca\textsuperscript{2+} sensitivity experiments, distinct Ca\textsuperscript{2+} solutions were prepared by adding to high-K\textsuperscript{+} solution appropriate amounts of CaCl\textsubscript{2} · 2H\textsubscript{2}O and 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) to obtain 0.5, 1.0, and 5.0 μM free Ca\textsuperscript{2+} concentrations, using the affinity constant value of 6.9 × 10\textsuperscript{6} M\textsuperscript{-1} (5). In the cell-attached experiments, all chemicals were added to the cells by perfusion of homogeneous solutions, and data were obtained after 3 min, so that the complete substitution of the volume of the chamber was guaranteed.

Chemicals. All chemicals were analytical grade. Penicillin, streptomyacin, HEPES, trypsin inhibitor, EGTA, bovine serum albumin, TEA\textsuperscript{+}, CsCl, MnCl\textsubscript{2}, digitonin, trypan blue dye, and fura 2-acetoxymethyl ester (AM) were purchased from Sigma (St. Louis, MO). Collagenase I (217 U/ml) was from Worthington Biochemical (Freehold, NJ), and pronase was from Boehringer (Mannheim, Germany). ANG II, [Lys\textsuperscript{2}]ANG II, and [1-sarcosine,8-b-alanine]ANG II (saralasin) were purified peptides 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 nonpeptide competitive inhibitors of ANG II (25), 2-n-butyl-4-chloro-5-hydroxymethyl-1–(2’–(1H-tetrazol-5-yl)-diphenyl-4-yl)methylimidazole (DUP-753 or losartan) and 1-imidazol[4,5-c]pyridine-6-carboxylic acid, 1–(4-dimethylamino)-3-methylphenyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-(5)-trifluoracetate(1:2) (PD-123319) were gifts from DuPont/Merck Pharmaceutical (Wilmington, DE). Other salts were from Merck (Darmstadt, Germany).

Recording techniques. Single-channel currents were recorded using either the inside-out or cell-attached modes of the patch-clamp technique (10). Patch electrodes were made of borosilicate glass (Garner Glass, Claremont, CA) through a two-stage puller (model PP-83, Narishige) and fire-polished (model MF-83 forge, Narishige) to a final pipette-tip resistance of 5–25 MΩ. A 1 M KCl-agar bridge connecting the Ag-Agl reference electrode was used to ground the bath solution. Both the cells and the electrode were visualized with an inverted microscope (Diaphot model, Nikon). Single-channel currents were captured and amplified through a patch-clamp amplifier (EPC7; List Electronics, Darmstadt, Germany) and stored on videotape (model PVC-6000, Philips-Hitachi, São Paulo SP, Brazil) through an analog-to-digital converter (model DR-384, Neuro-Corder; Neuro Data Instruments, New York, NY). Data were displayed on-line or from the videotape to a physiograph (model RS 3200; Gould, Cleveland, OH) and an oscilloscope (model MO 1221, Minipa, São Paulo SP, Brazil) via a low-pass filter (8-pole Bessel filter; Frequency Devices, Haverhill, MA) that was adequately adjusted. The indicated potentials refer to conventional membrane potential measurements, and, accordingly, outward currents were recorded as upward deflections. All experiments were done at room temperature.

Data acquisition and analysis. Usually, 10-min records were analyzed. Currents were filtered at 3 kHz and sampled at 1 kHz (1,000 μs/point) through a 16-bit analog-to-digital converter (TL-1 DMA interface; Axon Instruments, Foster City, CA) controlled by the Fetchex software (pCLAMP 5.1, Axon). The acquired records were analyzed using the computer program Transit version 1.0 (29), kindly provided by R. Latorre, Universidad de Chile. The duration and amplitude of each current level were determined using idealized records from the original data, constructed through the recognition of the transitions between distinct levels. Transitions were detected any time dI/dt (I = current amplitude) was higher than the slope threshold criterion, usually set at ±3σ of the mean baseline noise. The open probability of the channel (P\textsubscript{o}) was obtained by fitting the fraction of time the channel spent in a current level to a binomial distribution using the maximum likelihood procedure (software kindly supplied by O. Alvarez, Universidad de Chile). Alternatively, and mainly for the experiments with the peptides, the results are expressed as N P\textsubscript{o}, where N is the number of channels in the patch available to open. N P\textsubscript{o} values were calculated by the ratio between the mean current and the unitary single-channel current. The mean current (I\textsubscript{mean}) was obtained from the amplitude current distribution histogram, using the
following expression: \( I_{\text{mean}} = A_1 f_1 + A_2 f_2 + \ldots + A_n f_n \), where \( A_1, A_2, \) and \( A_n \) represent the area under the Gaussian curve for each current level \((f_1, f_2, f_n)\) present in the patch.

Selectivity experiments. The channel selectivity for Na\(^+\) or Cl\(^-\) over K\(^+\) was estimated in excised inside-out patches, by determining the reversal potentials and calculating the permeability ratio \( P_{\text{Na}}/P_{\text{K}} \) and \( P_{\text{Cl}}/P_{\text{K}} \). The pipette was filled with solutions containing Na\(^+\), and the internal solution was 120 mM NaCl plus 30 mM KCl or 30 mM KCl, according to the ion tested.

Voltage and Ca\(^{2+}\) sensitivity. The voltage current ratio of channel gating mechanism was studied through the \( P_{\text{Cl}}/P_{\text{K}} \) membrane potential relationship in inside-out or cell-attached experiments in the presence of high-K\(^+\) solutions. Data fitted to the Boltzmann distribution, and the voltage change necessary to cause an e-fold increase in the \( P_{\text{Cl}}/P_{\text{K}} \) (slope factor) and the membrane potential corresponding to 50% of the maximal \( P_{\text{Cl}}/P_{\text{K}} \) were determined.

[Ca\(^{2+}\)]\(_i\) measurements. Changes in [Ca\(^{2+}\)] were monitored fluorometrically using fura 2. The 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. The loading period with 2 \( \mu \)M fura 2-AM of 2.5-ml suspensions of freshly dispersed myocytes from the longitudinal layer of the guinea pig ileum (10\(^6\) cells/ml) in 150 mM KCl solution was 3 h at room temperature. After incubation, the excess fura 2-AM was removed by washing with six volumes of KCl solution and centrifugation at 715 \( \times \) g for 2 min. The cell pellet was then resuspended in the same solution for the fluorometric studies. The loading period with 2 \( \mu \)M fura 2-AM of 2.5-ml suspensions of freshly dispersed myocytes from the longitudinal layer of the guinea pig ileum (10\(^6\) cells/ml) in 150 mM KCl solution was 3 h at room temperature. After incubation, the excess fura 2-AM was removed by washing with six volumes of KCl solution and centrifugation at 715 \( \times \) g for 2 min. The cell pellet was then resuspended in the same solution for the fluorometric studies.

RESULTS

General properties of the channel. For hyperpolarized membrane potentials, unitary currents from excised inside-out membrane patches of freshly dispersed myocytes from the longitudinal layer of the guinea pig ileum, in the presence of symmetrical high-K\(^+\) solutions, displayed brief openings that were alternated with long periods of closures (up to several seconds). As membrane potential was gradually depolarized, the time the channel spent in the closed (nonconductive) state became shorter, its burst activity increased, and very fast and partial closures inside a long open dwell time (flickering activity) were more evident (not shown). The current-membrane potential relationship was linear in the range from −60 to 60 mV, the single-channel conductance was 257 ± 4 pS, and reversal potential was 0 mV (n = 19) (Fig. 1A). The gating mechanism of the channel was voltage dependent, with \( P_{\text{Cl}}/P_{\text{K}} \) increasing from −1 to −2% for −40 mV to 86% for 40 mV, with a \( V_{1/2} \) value of 16 mV and slope factor of 15 mV (Fig. 1B). The Ca\(^{2+}\) sensitivity of the channel was tested by manipulating the free [Ca\(^{2+}\)]. First, Ca\(^{2+}\) was chelated from the solution by adding 2 mM EGTA to the contaminant Ca\(^{2+}\)-free solution to 35 mV. These results indicate that the channel permeability to Cl\(^-\) or Na\(^+\) was very low compared with K\(^+\), with \( P_{\text{Cl}}/P_{\text{K}} \) values of 0.04 and 0.06, respectively.

The selectivity of the channel was investigated for Cl\(^-\) and Na\(^+\) in excised inside-out patches, by determining the reversal potential when the cellular face of the membrane patch was exposed to a bath solution containing 30 mM KCl, in the absence or presence of 120 mM NaCl. In both conditions, the reversal potential was shifted from 0 mV, in the presence of symmetrical high-K\(^+\) solution, to 35 mV. These results indicate that the channel permeability to Cl\(^-\) or Na\(^+\) was very low compared with K\(^+\), with \( P_{\text{Cl}}/P_{\text{K}} \) values of 0.04 and 0.06, respectively.

To further characterize this channel population present in myocytes of the guinea pig ileum, we examined the blockade by external Cs\(^+\) and TEA\(^+\), two well-known blockers of maxi-K\(^+\) channel in other tissues (18). As expected, 0.5 mM CsCl induced a significant decrease, −30%, in the single-channel current amplitude and channel activity in burst, which was alternated with long closed blocked dwell times (Fig. 1C). Similar results were obtained in the presence of 0.1 mM TEA\(^+\) (Fig. 1C). The blockade induced by TEA\(^+\) was concentration dependent, with a complete blockade of the channel activity with 1.0 mM TEA\(^+\). These two blockers, at the same concentrations as those tested at the extracellular face (0.5 mM CsCl and 0.1–1 mM...
TEA\textsuperscript{1}, did not change the channel activity when added in the bath solution (not shown).

Finally, we investigated the possibility of spontaneous changes in the channel gating (gearshift) and rundown phenomena, which occur with relative frequency for maxi-K\textsuperscript{1} channels in other tissues (18), by studying the channel activity during a 15-min recording period, in the presence of symmetrical high-K\textsuperscript{1} solutions at 20 mV. The experimental period was split into 2-min recording segments, which in turn were analyzed in fragments of 10 s. The \( P_0 \) calculated for the distinct 10-s period was compared with the \( P_0 \) obtained during the whole 2-min segment. As illustrated in Fig. 2 for the 5- to 7-min interval, the “partial” \( P_0 \) values were within the range 0.47 ± 0.02 (0.47 is the \( P_0 \) obtained for the total record and 0.02 is the SE of the partial \( P_0 \)). Because similar results were obtained for the other 2-min intervals studied, it was clear that the gating of this channel population presented neither gearshift nor rundown phenomena in the present experimental conditions. These channels were present in 90% of the patches studied, and usually there were from three up to six channels per patch. Occasionally, there was only one single channel in the patch.

Therefore, we may assume that the channel population observed in myocytes from the longitudinal layer of the guinea pig ileum is a member of the large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{1} channel membrane protein family (maxi-K\textsuperscript{1} channel), since its biophysical properties are consistent with the classical description for this channel.

Indirect activation of maxi-K\textsuperscript{1} channel by ANG II and [Lys\textsuperscript{2}]ANG II. We have observed that maxi-K\textsuperscript{1} channels are ubiquitous in myocytes of the guinea pig ileum, and, because their recruitment and modulation by distinct agonists have been described in other tissues (1, 4, 11, 17, 19), we investigated the effect of ANG II and its analog on these K\textsuperscript{1} currents. The experiments were performed in the cell-attached configuration with the myocytes bathed in high-K\textsuperscript{1} solution in the absence or presence of the agonist. At ~40 mV membrane potential, equipotent maximal concentrations of ANG

![Diagram](http://ajpcell.physiology.org/)

**Fig. 1.** Properties of the Ca\textsuperscript{2+}-activated K\textsuperscript{1} (maxi-K\textsuperscript{1}) channel in freshly-dispersed myocytes from the longitudinal layer of the guinea pig ileum. **A:** average single-channel current-membrane potential (\( I-V_m \)) relationship ranging from −60 to 60 mV and recorded from inside-out patches in symmetrical high-K\textsuperscript{1} solutions \((n = 19)\). Data are presented as means, and SE are shown when SE bars are larger than the symbols. Data were fitted by linear regression \((r = 0.99)\). **B:** voltage and Ca\textsuperscript{2+} dependence of the gating mechanism of the channel. Open probability \((P_0)\)−\( V_m \) relationships in the presence of symmetrical high-K\textsuperscript{1} solutions \(\bullet; n = 19\) and in the presence of 2 mM EGTA in the bath solution \(\triangle; n = 5\). Data are presented as means, and SE are shown when SE bars are larger than the symbols. Continuous lines represent the fitting of the data: in the absence of EGTA, lines were made with the use of the Boltzmann equation \( P_0 = P_{max}[1 + \exp(V_m - V_{1/2})/\text{slope factor}]^{-1} \), where \( P_{max} \) (maximum \( P_0 \)) = 0.87, \( V_{1/2} \) (membrane potential corresponding to 50% of the maximal \( P_0 \)) = 16 mV, and slope factor = 15.5 mV; and, in the presence of EGTA, lines were made by eye. **C:** blockade of maxi-K\textsuperscript{1} channel currents by external Cs\textsuperscript{1} and tetraethylammonium chloride (TEA\textsuperscript{1}). Outward single-channel current recorded in 3 different inside-out patches, with the pipette filled with high-K\textsuperscript{1} solution (control) or with high-K\textsuperscript{1} solution plus 0.5 mM Cs\textsuperscript{1} or 0.1 mM TEA\textsuperscript{1}. Membrane potential of the patches was 40 mV. Arrows at right indicate 0 current level.
II (n = 15) and [Lys²]ANG II (n = 10) promoted a significant and sustained, at least for 5 min, increase of the channel activity in almost all patches tested, by increasing the $P_0$ value and/or the number of channels simultaneously activated in the patch (Fig. 3). Neither the channel conductance nor the reversal potential (not shown) was affected by these peptides. To identify the channel population activated by ANG II, the patches were routinely excised at the end of the cell-attached experiments and the conductance, voltage, and Ca²⁺ dependence were similar to those described for the maxi-K⁺ channel in the previous item. As far as we know, there are no reports in the literature of activation of maxi-K⁺ channels by ANG II in smooth muscle. So far, our result contrasts with the negative modulation of this channel population by ANG II in vascular and airway smooth muscles (20, 28). The increased channel activity promoted by ANG II or [Lys²]ANG II was either blocked by 10⁻⁶ M saralasine or 10⁻⁶ M DUP-753 added to the bath solution (Fig. 3A) but not by PD-123319 (not shown). These results indicate that 1) the channel activation promoted by these peptides is primarily due to their binding to the AT₁ receptor subtype (26) and 2) the maxi-K⁺ channel population must be indirectly activated through the production of at least one second messenger because the modulation of the channel activity by ANG II was observed in cell-attached patch experiments and the peptide was added in the bath solution.

Activation of the maxi-K⁺ channel by ANG II was concentration dependent. Figure 4 shows the $NP_0$ measured 3 min after exposure to the indicated ANG II concentrations normalized to the $NP_0$ observed in the absence of the peptide in the bath solution. Concentration values lower than 10⁻⁹ M ANG II did not induce any significant increase of the $NP_0$ values compared with the control. The maximum $NP_0$ value was reached with 10⁻⁷ M, and the ANG II concentration resulting in half-maximum activation (EC₅₀) was 10 nM. These values are close to the maximal concentration and EC₅₀ value described for the contractile response of the isolated guinea pig ileum to ANG II (23), suggesting that the membrane signaling during ANG II action is not impaired when the cell membrane is kept depolarized.

Fig. 2. Time independence of channel activity. A: single-channel outward currents recorded from an excised inside-out patch in the presence of symmetrical high-K⁺ solutions at intervals of 2–4, 5–7, and 10–12 min. Membrane potential = 20 mV. B: distribution of partial $P_0$ calculated for 10-s fragment recordings (■) around the total $P_0$ (continuous line) obtained from the 5- to 7-min interval. Total $P_0$ was 0.47, and partial $P_0$ values were within 0.47 ± 2σ interval (dashed lines).

Fig. 3. A: effect of ANG II antagonists on the indirect positive modulation of maxi-K⁺ channel activity by ANG II and its synthetic analog [Lys²]ANG II. Records were from cell-attached patches in the presence of high-K⁺ solution in the bath and pipette, and 10⁻⁶ M DUP-753 or 10⁻⁶ M saralasine was added to the external solution 5 min after the activation of single-channel currents by 10⁻⁷ M ANG II. Patches were submitted at −40 mV membrane potential. $NP_0$ (where $N$ is the number of channels) values were 0.019, 1.044, and 0.069 for the control condition, ANG II-induced maxi-K⁺ activation, and further blockade by DUP-753, respectively. $NP_0$ values were 0.001, 1.589, and 0.008 for the control condition, [Lys²]ANG II-induced maxi-K⁺ activation, and further blockade by saralasine, respectively. Arrows at right indicate 0 current level. Records were representative of 4 experiments for each peptide. B: $P_0$-time curve in the absence and after addition of 10⁻⁷ M ANG II added to the bath solution for the experiment illustrated in A.
activated by ANG II.

scribed for the maxi-K channel indirectly activated by ANG II at $V_m$ of $-40$ mV. $N_P_o$ were measured 3 min after exposure of the cell to the indicated concentrations of ANG II normalized to the $N_P_o$ observed in the absence of the peptide from the bath solution. Each point represents the mean, and vertical bars represent SE. Number of cases studied is indicated in parentheses for each value of ANG II concentration. Data were fitted using a sigmoidal function, with a Hill coefficient of 1.

Voltage and Ca$^{2+}$ dependence of maxi-K$^+$ channel activation by ANG II. The $P_o$-$V_m$ relationship of the maxi-K$^+$ channel in the presence of $10^{-7}$ M ANG II or $10^{-6}$ M [Lys$^2$]ANG II was shifted to more negative membrane potentials but presented the same slope factor as the one obtained in the absence of the peptides (Fig. 5). This suggests that the putative second messenger responsible for the maxi-K$^+$ channel activation induces no modification of the voltage sensitivity of its gating mechanism. The most obvious candidate as second messenger is the intracellular Ca$^{2+}$, the primary regulator of this channel. This assumption is based on the similar shift between the $P_o$-$V_m$ membrane potential relationship by increases of intracellular Ca$^{2+}$ described for the maxi-K$^+$ channel in other tissues (18) and that observed in the presence of ANG II in cell-attached experiments (Fig. 5) and on the fact that ANG II induces Ca$^{2+}$ entry (24), leading to increased Ca$^{2+}$ concentration at the intracellular microenvironment of maxi-K$^+$ channels. To test this possibility, we have studied the indirect effect of ANG II on K$^+$ currents in the absence of external Ca$^{2+}$. Removal of external Ca$^{2+}$ by addition of 2 mM EGTA completely abolished the enhanced activation of maxi-K$^+$ channel by $10^{-7}$ M ANG II in cell-attached membrane patches (Fig. 6). A direct piece of evidence for the role of intracellular Ca$^{2+}$ as second messenger was obtained by measuring free [Ca$^{2+}$]$_j$ in fura 2-loaded, high-K$^+$ depolarized intestinal myocytes stimulated by ANG II peptides. A maximal concentration of either ANG II (Fig. 7) or [Lys$^2$]ANG II (not shown) induced a fast and sustained increase of cytosolic Ca$^{2+}$ concentration. For the cells stimulated with ANG II, [Ca$^{2+}$]$_j$ values were significantly higher than the basal free [Ca$^{2+}$], (500 nM) in its absence (Fig. 7, inset) and correspond to a $37 \pm 5\%$ ($n = 4$) increase. Although statistical significance was not obtained for the increase in free [Ca$^{2+}$], induced by [Lys$^2$]ANG II (Fig. 7, inset), the $37 \pm 8\%$ ($n = 4$) increase promoted by this analog cannot be ignored, since both peptides enhanced the channel activity in cell-attached experiments in a comparable intensity. These data suggest that the [Ca$^{2+}$], increase was high enough to explain the K$^+$ currents observed through maxi-K$^+$ channels at the cell-attached level in freshly dispersed myocytes of the guinea pig ileum bathed in high-K$^+$ solution, as described in Fig. 3.

DISCUSSION

In this study, we have identified the presence of high-conductance, Ca$^{2+}$-dependent, and voltage-dependent K$^+$ channels (maxi-K$^+$ channel) in the plasma

![Graph](image-url)
The quite high, but stable, basal fluorescence values of the myocytes loaded with fura 2 during the control period might be attributed to Ca\(^{2+}\) binding to cytosolic nonfluorescent proteins (23), where (23) is also respected in this experimental condition. Indeed, an equipotent dose of [Lys\(^2\)]ANG II resulted in similar activation of the maxi-K\(^+\) channel (Fig. 3) and [Ca\(^{2+}\)] increases (Fig. 7).

Previous studies demonstrated that ANG II can modulate different ionic conductances in distinct tissues. In renal artery myocytes, ANG II was shown to inhibit the delayed rectifier K\(^+\) current (7) via a signal transduction pathway involving protein kinase C activation (3) and to activate a nonspecific cationic conductance in ear arterial cells from the rabbit (12) or in cultured myocytes from the longitudinal layer of the guinea pig ileum (21). The activation of the maxi-K\(^+\) channel by ANG II in freshly dispersed guinea pig longitudinal myocytes contrasts with the blocking effect exerted by this peptide on this channel population from porcine coronary artery cells (20) or plasma membrane vesicles from this tissue incorporated in lipid bilayers (28). The blockade was attributed either to a direct action, with no interference of G protein or other second messenger (28), or to an indirect action through an unknown second messenger system, independent of protein kinase C or tyrosine kinase activation (20). However, the positive modulation of the maxi-K\(^+\) channel by ANG II, described here, is consistent with the rise in [Ca\(^{2+}\)], ~37%, above the basal [Ca\(^{2+}\)] in fura 2-loaded myocytes (Fig. 7), which was high enough to guarantee an increase of the Ca\(^{2+}\) concentration in the microenvironment of the channels and accounted for the shift of the P\(_o\)-voltage relationship to more negative membrane potentials (Fig. 5). The quite high, but stable, basal fluorescence values of the myocytes loaded with fura 2 during the control period might be attributed to Ca\(^{2+}\) influx through nonselective cation channels (21) or voltage-gated Ca\(^{2+}\) channels activated by membrane depolarization, since the cells were maintained in high-K\(^+\) solution throughout the experimental period, including the loading step of the myocytes with fura 2. In fact, voltage-damp studies on voltage-gated Ca\(^{2+}\) channels in smooth muscle cells from various tissues have revealed that, during prolonged membrane depolarization, a popul-
tion of voltage-gated Ca\textsuperscript{2+} channels are not inactivated and they allow sustained influx of extracellular Ca\textsuperscript{2+} (6, 13). The distinct effect induced by ANG II on maxi-K\textsuperscript{+} channel activity described here cannot be attributed to the elevated basal Ca\textsuperscript{2+}, since the reported inhibition of maxi-K\textsuperscript{+} channels in porcine coronary artery cells was observed also when cells were bathed in high-K\textsuperscript{+} solution and in the presence of the Ca\textsuperscript{2+} ionophore A-23187 (20), two conditions likely to promote elevated [Ca\textsuperscript{2+}]. This variability shows the great complexity of the signal transduction system triggered by this peptide, which might be distinct in vascular and visceral smooth muscles, although involving the same receptor subtype. In general, three possible sources of Ca\textsuperscript{2+} are available for activation of the maxi-K\textsuperscript{+} channel: 1) extracellular Ca\textsuperscript{2+}, 2) intracellular Ca\textsuperscript{2+} or Ca\textsuperscript{2+} stores, and 3) a combined source. Modulation of maxi-K\textsuperscript{+} channels by several excitatory agents has been reported in distinct tissues. In rabbit colonic smooth muscle, substance P causes activation of maxi-K\textsuperscript{+} channels, due to Ca\textsuperscript{2+} entry through dihydropyridine-sensitive channels (19); in tracheal smooth muscle cells, acetylcholine mediates this effect by depleting intracellular Ca\textsuperscript{2+} stores (30). Endothelin-1 exerts a dual effect on the maxi-K\textsuperscript{+} channels, with low doses stimulating maxi-K\textsuperscript{+} channels and higher doses provoking a steady inhibition (11). Although our results do not allow us to identify the Ca\textsuperscript{2+} source used by ANG II, some considerations may favor one of the three possibilities. With regard to the extracellular Ca\textsuperscript{2+} source, the contractile response of this tissue is mainly due to Ca\textsuperscript{2+} influx through the plasma membrane (22), which is due to depolarization (24) and opening of voltage-dependent Ca\textsuperscript{2+} channels; ANG II increases both \textsuperscript{24}Na\textsuperscript{+} and \textsuperscript{45}Ca\textsuperscript{2+} uptake (25) and indirectly modulates a nonsensitive cation channel in the guinea pig ileum (21). We have now demonstrated that activation of the maxi-K\textsuperscript{+} channel by externally applied ANG II was prevented by chelation of extracellular Ca\textsuperscript{2+} with EGTA (Fig. 6). With regard to the intracellular store, ANG II induces a stimulation of IP\textsubscript{3} production (16), which would activate Ca\textsuperscript{2+}-permeable channels from the sarcoplasmic reticulum. Therefore, so far, the most plausible alternative seems to be the third one. This is reinforced by the sustained increase in both maxi-K\textsuperscript{+} channel activity and [Ca\textsuperscript{2+}], which might be interpreted as a consequence of emptying and refilling of Ca\textsuperscript{2+} stores through Ca\textsuperscript{2+} influx pathways (15). Obviously, these conclusions must be regarded with caution, since an inhibition by ANG II of the ATP-driven Ca\textsuperscript{2+} pump in the plasma membrane (2) or other Ca\textsuperscript{2+} extrusion mechanism would also induce a steady [Ca\textsuperscript{2+}] increase. In conclusion, freshly dispersed myocytes from the longitudinal layer of the guinea pig ileum are a fruitful and inviting model for further investigations of the pharmacomechanical coupling of vasoactive peptides and the mechanisms underlying desensitization and tachyphylaxis by ANG II in this tissue.

We are grateful to Dr. Ramon Latorre for suggestions and criticisms, to Dr. Maria Etsuko M. Oshiro and Dr. Alice T. Ferreira for support in the intracellular Ca\textsuperscript{2+} measurements, and to Nelson A. Mora for technical assistance.

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo. F. Romero was on a fellowship from CNPq, and B.A. Silva was on a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

Present addresses: F. Romero, Depto de Cs. Preditencias, Facultad de Medicina, Universidad de La Frontera, Temuco, Chile; B. A. Silva, Laboratório de Tecnologia Farmacêutica, Universidade Federal do Pará, Paraíba, Brazil.

Address for reprint requests: J. Aboulafia, Universidade Federal de São Paulo, Escola Paulista de Medicina, Caixa Postal 20, 04034-970 São Paulo SP, Brazil.

Received 15 August 1997; accepted in final form 1 December 1997.

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