Calcium-Sensitive Chloride Channels in Vascular Smooth Muscle Cells (43853)

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Abstract. **Chloride (Cl^-) channels were characterized in vascular smooth muscle cells (VSMC) using radioisotope flux and patch-clamp electrophysiological techniques. Transmembrane ^{125}iodine (^{125}I) efflux from subcultured (Passage 1–5) rat aortic VSMCs was used as an indicator of Cl^- movements to study the relationship between intracellular calcium concentration ($[Ca^{2+}]_i$) and Cl^- channel activity. Angiotensin II (Ang II) (10^{-7} *M*) and adenosine 5′-triphosphate (ATP) (10^{-4} *M*) induced rapid increases (9.7- and 14.9-fold, respectively) in ^{125}I efflux rates. We found that both Ang II- and ATP-stimulated ^{125}I efflux and $[Ca^{2+}]_i$ increases were completely abolished after brief incubation (20 μM, 20 min) with the acetoxymethyl ester of 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM), a membrane-permeable Ca^{2+} chelator. However, when external EGTA was used to blunt agonist-stimulated Ca^{2+} influx, ^{125}I efflux was still increased in response to Ang II and ATP. These data suggest that Ca^{2+} release from intracellular sites is sufficient to activate Cl^- channels in response to Ang II and ATP. Using standard patch-clamp electrophysiological techniques, we found that Ang II, a Ca^{2+}-mobilizing agonist, stimulated outward Cl^- currents (g_{Cl} = 75 pS) in cell-attached (C/A) patches of primary and subcultured VSMCs. Collectively, these data suggest that Ang II and other vasoconstrictor agents stimulate Cl^- channel activity via increases in $[Ca^{2+}]_i$. Cl^- channel activation may help to depolarize the VSMC membrane leading to increased Ca^{2+} influx during agonist stimulation.** [P.S.E.B.M. 1995, Vol 208]

At least four distinct pathways contribute to chloride (Cl^-) homeostasis in vascular smooth muscle cells (VSMC): (i) $Na^+/K^+/Cl^-$ cotransport (1); (ii) Na^+-dependent Cl^-/HCO_3^- exchange (2–3); (iii) Na^+-independent Cl^-/HCO_3^- exchange (4); and (iv) conductive Cl^- channels (5–9). $Na^+/K^+/Cl^-$ cotransport is believed to be important in cell volume regulation (10). Loop diuretics, such as furosemide and bumetanide ($Na^+/K^-/Cl^-$ cotransport inhibitors), block ^{36}Cl influx in rabbit aorta (11), ouabain-insensitive ^{86}Rb influx in rat and rabbit aorta (12), and ^{86}Rb influx in cultured rat aortic VSMC (1). In rabbit aorta, furosemide induces a substantial decrease in resting steady state ^{36}Cl content, suggesting that the cation-coupled Cl^- cotransporter is involved in maintaining basal VSMC $[Cl^-]_i$ (11). Both Na^+-dependent and -independent Cl^-/HCO_3^- exchangers have been identified in VSMCs (2–4) and are involved in intracellular pH regulation (13). 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an anion exchanger inhibitor, reduces basal ^{36}Cl efflux (in HCO_3^- buffer) by approximately 30% and 60% in rat aorta and femoral artery, respectively (14). Similarly, DIDS inhibits ^{36}Cl efflux/influx in intact rabbit aorta (11).

Ligand- and voltage-gated Cl^- channels have been characterized extensively in neurons (15), epithelial cells (16), and skeletal muscle (17). In addition to the ligand-gated Cl^- channels, molecular cloning methods have identified at least seven other Cl^- channel subtypes (18). Electrophysiological techniques have been used to identify at least five different Cl^- currents in

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cardiac myocytes, and evidence suggests that Cl^- channels play an important function in cardiac tissue by modulating action potential generation (18). Ca^{2+}-activated Cl^- channels have been described in a variety of cell types, including parotid acinar cells (19), lacrimal glands (20), epithelial cells (16), mast cells (21), platelets (22), and neutrophils (23). In contrast, little information is available concerning the role of Cl^- channels in the regulation of VSMC membrane potential in intact arteries. Harder and Sperelakis (24) reported that replacement of extracellular Cl^- with either NO_3^- or glutamate only transiently depolarized guinea pig superior mesenteric artery, suggesting that it did not contribute to steady state resting membrane potential. However, Goligorsky and co-workers (25–26) have recently demonstrated that endothelin-induced contraction of rat aortic rings, as well as vasoconstriction of the rat renal microcirculation, can be inhibited by indanyloxyacetic acid, a putative Cl^- channel blocker. In addition, recent reports have provided evidence that ATP, noradrenaline, and endothelin stimulate Cl^- currents in freshly isolated VSMCs (5–7). The goal of the present study was to investigate which Cl^- channel types are expressed by rat aortic VSMCs in short-term culture, as well as to test their Ca^{2+} and agonist sensitivity. In particular, we report using a combination of patch-clamp techniques and ^{125}iodine (^{125}I) efflux measurements that Cl^- channels in these cells are activated in response to Ang II and ATP in a Ca^{2+}-dependent manner. Therefore, cultured VSMCs may serve as a stable model for future molecular studies aimed at understanding the relationship between Cl^- channel regulation/expression and abnormal VSMC function in hypertension.

Materials and Methods

Cell Culture. Primary cultures of VSMCs were established from pooled rat aortas (male Sprague-Dawley; 200–250 g) using enzymatic dissociation techniques and were propagated as described previously (27). Primary and passaged cells (Subculture 1–5) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

^{125}Iodine Efflux Studies. ^{125}Iodine (^{125}I) was chosen as a marker for Cl^- channel activity in these studies for several reasons: (i) it has a higher specific activity than ^{36}Cl; (ii) it is transported poorly by the $Na^+/K^+/Cl^-$ cotransporter in VSMCs and by anion exchangers (1, 28); and (iii) its permeability through anion-specific channels is greater than that of Cl^- (8). Cultured VSMCs were seeded onto 35-mm culture dishes and grown to 90% confluence (≈7 days). Next, cells were exposed to 6 μCi/ml ^{125}I (carrier-free, DuPont NEN) for 12–18 hr in complete growth media. On the following day, the radioactive media was aspirated, and the cells were washed with a standard efflux buffer (in m*M*): 140 NaCl, 10 HEPES, 3.33 KH_2PO_4, 0.83 K_2HPO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose). At time zero, 1 ml of buffer was added to the dish for 3 min. Subsequently, 1 ml of efflux media was removed every 20 sec and replaced with 1 ml of fresh buffer. Using this buffer replacement protocol, samples were obtained for a 3-min control period. Cells were then exposed to agonist (dissolved to the appropriate concentration in efflux buffer), and sample collection continued every 20 sec for an additional 5 min. Cell-associated ^{125}I radioactivity remaining at the end of the experiment was extracted with 1.0% SDS/0.1% NaOH and was quantified using a Searle (Woodale, IL) gamma counter (Model 1285). Total intracellular counts at the beginning of the experiment were estimated by back-adding counts collected during each sample period. ^{125}I efflux rates (% counts effluxed/min) were determined for each 20-sec period by dividing the number of counts effluxed by the total intracellular counts present at the beginning of that sample period.

Measurement of Ca^{2+}-Sensitive Fura-2-Fluorescence. Fura-2, a Ca^{2+}-sensitive fluorescent dye (Molecular Probes, Inc., Eugene, OR), was used to monitor changes in $[Ca^{2+}]_i$ in VSMC suspensions as described by Brock and Capasso (29). Cytosolic Ca^{2+} concentrations were estimated using the formula: $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) \cdot (EGTA_{380}/DIG_{380})$ where R was the fluorescence ratio within the cell, K_d was 224 n*M* (15), $EGTA_{380}$ was the fluorescence signal (380 nm) in the presence of 10 m*M* EGTA (pH = 10), and DIG_{380} was the fluorescence signal following digitonin addition in the presence of 1.5 m*M* $CaCl_2$. Fluorescence tracings depicting $[Ca^{2+}]_i$ recordings are typical tracings and are representative of at least three independent experiments.

Patch-Clamp Recording Techniques. Single-channel Cl^- currents were recorded using standard patch-clamp techniques as described by Hamill *et al.* (30). Pipettes were pulled from 8161 type glass capillary tubing (Garner Glass, Co., Claremont, CA) using a two-stage electrode puller Model PB-7; Narashige, Tokyo, Japan). The taper of the glass was insulated with Q-Dope, and the tips were fire-polished immediately prior to the experiment to produce a pipette resistance of 2 to 5 MΩ when the lumen was filled with recording solution. The reference electrode (Ag/AgCl) was connected to the bath by an agar-KCl (0.1 *M*) bridge. A Nikon Diaphot inverted microscope with Hoffman modulation optics (×400 magnification) was used to visualize the cells.

All patch clamp experiments were conducted at room temperature (20°C), and, unless otherwise stated, the following solution was used in the pipette and bath (in m*M*): 150 NaCl; 1 $MgCl_2$; 5 HEPES; 1 EGTA; pH 7.2. Cells were seeded onto plastic cover-

slips which had been previously coated with a sterile 0.001% solution of collagen (Type IV, Sigma Chemical Co., St. Louis, MO) and placed in a flow-through chamber mounted on the microscope stage. Chamber fluid (0.5 ml) was changed using a peristaltic pump at a rate of 2.5 ml/min. After 10 GΩ patch seals were obtained in the cell-attached (C/A) recording configuration, holding voltages (V_h) were varied between ±50 mV to determine if there were Cl^- channel openings and closings in the patch. If Cl^- channels were present, currents were recorded for 20–25 sec at various V_hs (±80 mV/20 mV increments). Single-channel recordings were filtered at a cutoff frequency of 3 kHz. Currents measured under these conditions were stored on video tape using a Toshiba model 200T PCM video recorder for later analysis. In control experiments, NaCl was replaced by N-methyl-D-glucamine (NMDG)-Cl to eliminate the potential contribution of Na^+ currents to channel activity in I/O patches.

The ability of Ang II, a Ca^{2+}-mobilizing agonist in cultured rat aortic VSMCs (27), to stimulate Cl^- channel activity was tested in intact cells using the C/A recording configuration. Due to the transient activation of ^{125}I efflux in response to Ang II, V_h was held at −50 mV in C/A patches, and channel activity was recorded continuously at this potential. We chose to record current activity at −50 mV since we and others (9) have found this value to be near the resting membrane potential. After an initial control recording period (1–2 min), Ang II was added to the bath yielding a final concentration of 10^{-7} *M*, and channel activity was recorded in the presence of the Ang II bolus for an additional 4–5 min. To analyze Cl^- channel activity, data tapes were played back, low-pass filtered and analyzed using commercially available computer software (pClamp 5.5.1, Axon, Inc., Foster City, CA). Single Cl^- channels were characterized by analyzing percentage of open and closed times, single-channel conductance (g_{Cl}), and open-state probability (NP_o).

Statistical Analysis. Descriptive statistics reported include mean and standard error of mean (SEM). Results were analyzed by analysis of variance (ANOVA). The confidence level for comparison was $P < 0.05$.

Results

^{125}Iodine Efflux Studies. Both primary and passaged VSMCs exhibited constant ^{125}I efflux rates under basal conditions (Fig. 1a and Table I). As illustrated in Figure 1b, Ang II (10^{-7} *M*) caused a rapid, transient increase in ^{125}I efflux above basal values (9.7- ± 1.4-fold increase; $n = 16$) which returned to control levels after approximately 1–2 min. Similar results were obtained when VSMCs were challenged with ATP (10^{-4} *M*) (14.9- ± 1.1-fold increase; $n = 6$) (Fig. 1c). Since both agonists stimulate increases in $[Ca^{2+}]_i$,

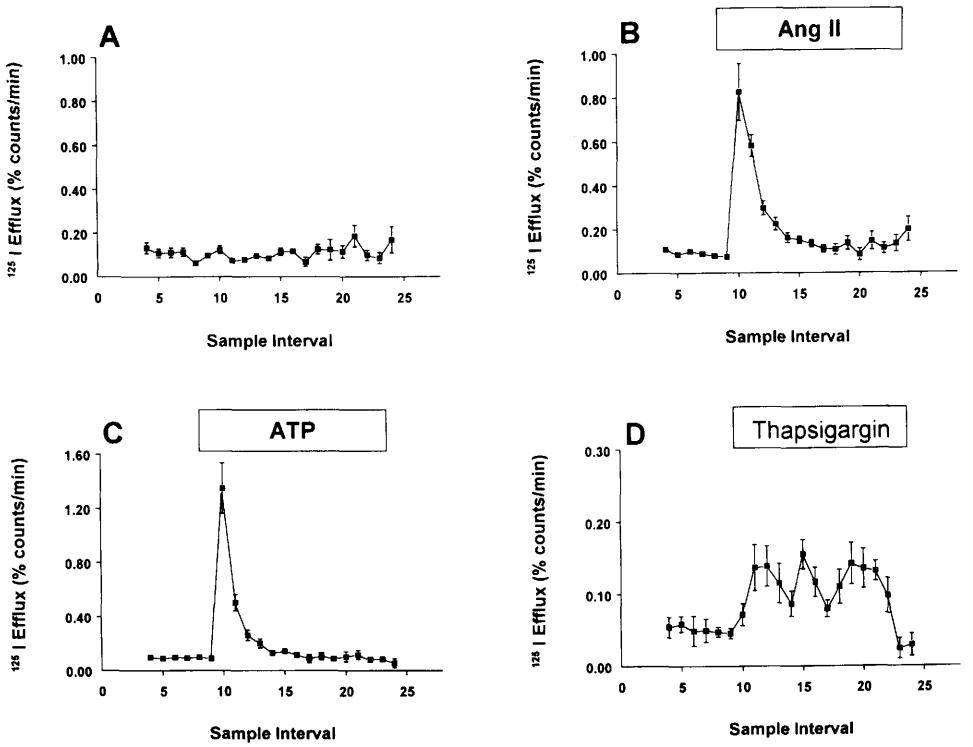

Figure 1. Effect of different agonists on ^{125}I efflux in cultured VSMCs. Cultured cells (Passage 1–5) were equilibrated in efflux buffer, exposed to buffer containing no additions (Panel A), 10^{-7} *M* Ang II (Panel B), 10^{-4} *M* ATP (Panel C) or 10^{-6} *M* thapsigargin (Panel D) and ^{125}I efflux continuously sampled over Interval 10–24. ^{125}I efflux rates were calculated as described in Materials and Methods. Each sample interval corresponds to a 20-sec incubation period. Total duration of the experiment was 8 min. Data are expressed as the mean ± SEM for $n = 13$ (Panel A), $n = 16$ (Panel B), $n = 6$ (Panel C) and $n = 4$ (Panel D) experiments.

Table I. Effect of Different Treatments on Mean ^{125}I Efflux Rates

Treatment	Mean ^{125}I efflux (% counts effluxed/min)	SEM		*n*
Control	0.113	0.008		13
Ang II/BAPTA	0.090	0.013		7
Control/BAPTA	0.072	0.007	*	5
Control/EGTA	0.063	0.005	**	5
Forskolin	0.135	0.007		7
DIDS	0.058	0.010	*	5
Bumetanide	0.092	0.006		5

Note. Means represent the average ^{125}I efflux calculated over Sampling Intervals 10–24 (*$P < 0.05$; **$P < 0.01$). Studies were done using passaged VSMCs (Passage 1–5). The concentrations of agents were Ang II (10^{-7} *M*); BAPTA (20 μ*M*, 30 min); forskolin (2.5 μ*M*); DIDS (50 μ*M*); and bumetanide (10^{-4} *M*).

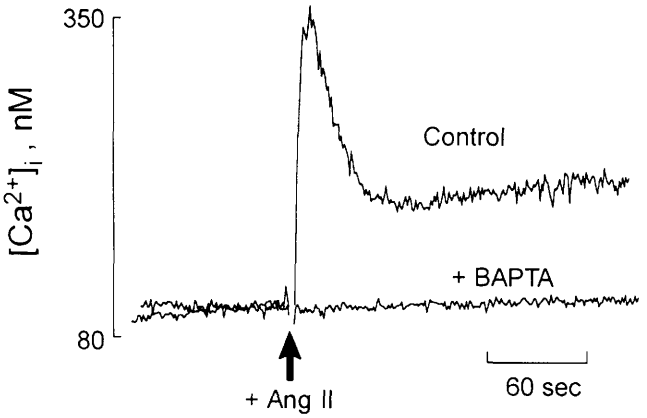

Figure 3. Effect of BAPTA loading on Ang II-stimulated $[Ca^{2+}]_i$ in VSMCs. In control experiments, Ang II (10^{-7} *M*) induced a 4- to 5-fold increase in $[Ca^{2+}]_i$. Fura-2 loaded VSMCs were exposed to BAPTA-AM (20 μ*M*) immediately prior to resuspending cells in the cuvette. Fluorescence tracings depicting $[Ca^{2+}]_i$ recordings are typical tracings and are representative of at least three separate experiments.

we tested the hypothesis that ^{125}I efflux was sensitive to changes in $[Ca^{2+}]_i$. Thapsigargin (10^{-6} *M*) stimulated an increase in $[Ca^{2+}]_i$ (peak response = 238 ± 12 n*M*) as measured by fura-2 fluorescence and induced approximately a 3-fold increase in ^{125}I efflux above basal levels (Fig. 1d). The time course for thapsigargin-stimulated Ca^{2+} mobilization (data not shown) was closely related to its ^{125}I efflux profile. When VSMCs were exposed to the Ca^{2+} ionophore, ionomycin (5×10^{-6} *M*), a rapid increase in ^{125}I efflux occurred which was similar in magnitude to that seen in response to Ang II or ATP (data not shown). Next, 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetra acetic acid (BAPTA) was used as a cell Ca^{2+} buffer to dampen agonist-stimulated increases in $[Ca^{2+}]_i$. Incubation of VSMCs with the acetoxymethyl ester of BAPTA (20 μ*M*, 20 min) completely inhibited Ang II-stimulated ^{125}I efflux ($n = 7$) (Fig. 2a). Under similar incubation conditions, BAPTA-AM loading prevented Ang II- (Fig. 3) and ATP (data not shown)-stimulated $[Ca^{2+}]_i$ transients. However, when Ca^{2+} was removed from the efflux buffer and replaced with 2 m*M* EGTA, the peak Ang II-stimulated ^{125}I efflux remained unchanged (Fig. 2b). These results support the hypothesis that intracellular Ca^{2+} release is sufficient to initiate agonist-stimulated ^{125}I efflux. Both BAPTA-AM treatment and extracellular Ca^{2+} removal caused basal ^{125}I efflux rates to decrease in the absence of Ang II (Table I).

Since Na^+-dependent and -independent Cl^-/HCO_3^- transport mechanisms are inhibited in HCO_3^--free efflux media (13), our data indicate that these particular transporters do not mediate agonist-stimulated ^{125}I efflux. To test the potential role of other Cl^- transport mechanisms in mediating agonist-stimulated ^{125}I efflux, VSMCs were pretreated with either bumetanide (100 μ*M*, 30 min) or DIDS (50 μ*M*, 10 min). Bumetanide, an inhibitor of $Na^+/K^+/Cl^-$ cotransport, did not affect basal ^{125}I efflux (Table I). In VSMCs challenged with Ang II, bumetanide delayed slightly the onset of the agonist-stimulated ^{125}I efflux, but did not affect the peak efflux response (Fig. 4a). In

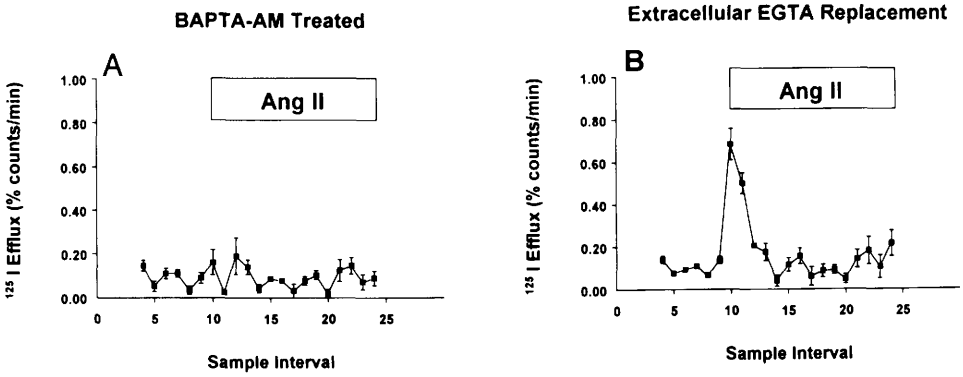

Figure 2. Effect of Ca^{2+}-chelators on Ang II-stimulated ^{125}I efflux. (A) Pretreatment of VSMCs with BAPTA-AM (20 μ*M*/20 min, $n = 7$) inhibited Ang II-stimulated ^{125}I efflux. (B) Extracellular Ca^{2+}-chelation (2 m*M* EGTA was substituted for Ca^{2+} in the efflux buffer) had no effect on Ang II-stimulated ^{125}I efflux ($n = 7$). ^{125}I efflux rates were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM.

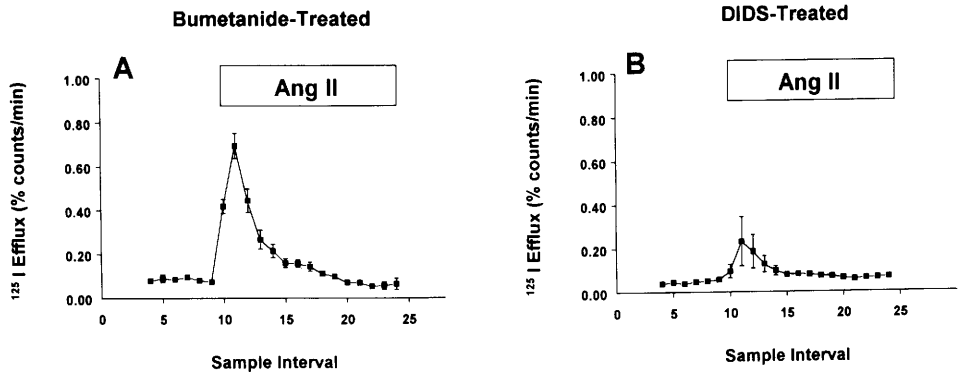

Figure 4. Effect of Cl^- transport blockers on Ang II-stimulated ^{125}I efflux. (A) VSMCs were incubated with the $Na^+/K^+/Cl^-$ inhibitor, bumetanide (10^{-4} *M*, 30 min), prior to adding 10^{-7} *M* Ang II (n = 5). (B) The Cl^- channel inhibitor, DIDS (5×10^{-5} *M*) was added 10 min prior to adding Ang II (n = 5). ^{125}I efflux rates were calculated as described in Materials and Methods. Data are expressed as the mean ± SEM.

contrast, when VSMCs were pretreated with DIDS, a Cl^- channel blocker in VSMCs (31), Ang II-stimulated ^{125}I efflux was inhibited by approximately 67% (Fig. 4b). In other experiments, we tested the effects of cyclic AMP, an activator of epithelial cell Cl^- channels (16), on ^{125}I efflux using forskolin. Addition of 2.5 μ*M* forskolin did not augment ^{125}I efflux from VSMCs (Table I).

Patch-Clamp Studies. Under C/A recording conditions, outwardly directed Cl^- currents could not be differentiated from outward K^+ currents; however, inward currents represented Cl^- channel activity. When $NMDG^+$ was substituted for Na^+ in buffer solutions, no difference in the current-voltage relationship was noted (data not shown) suggesting that inward, positive currents were due solely to the outward movement of negatively charged chloride ions. Angiotensin II (10^{-7} *M*) induced a rapid increase in Cl^- channel activity (Fig. 5) whose time of onset was similar to Ang II-stimulated ^{125}I efflux. Ang II dramatically increased the percentage of open time of the Cl^- channel (control: 2.6% ± 0.8% versus Ang II: 83.4% ± 3.2%). While current amplitude was unaffected by Ang II treatment, open state probability (NP_o: where N = number of channels and P_o = probability that a channel is open) increased from 0.043 ± 0.013 under control conditions to 0.927 ± 0.036 during the Ang II exposure period. The point conductance of the Cl^- channel at V_h = −50 mV was 75 pS. Ang II-stimulated Cl^- channel activity in C/A patches consistently decayed within 2 min. Open time histograms for Cl^- channel activity were constructed using analysis software and demonstrated a significant increase in the open time constant (τ_o) for the Cl^- channel after exposure to Ang II (Fig. 6).

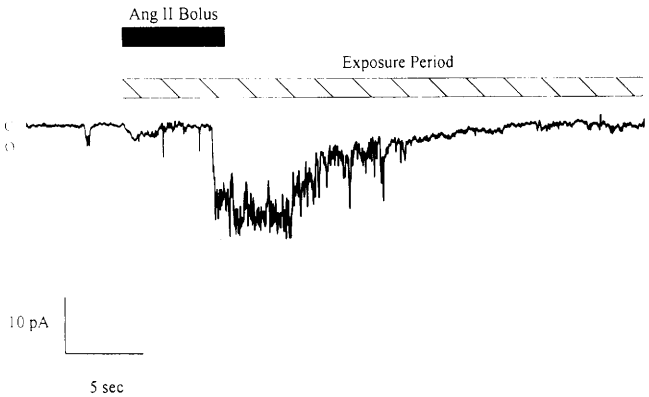

Figure 5. Effect of Ang II on Cl^- channel activity recorded in a cell-attached patch. Cl^- current was recorded continuously prior to and following bolus addition of Ang II. The black bar denotes the time interval during which the Ang II bolus (final concentration = 10^{-7} *M*) was added to the bath and the diagonal bar represents the duration of Ang II exposure. Holding potential was set to −50 mV in this experiment. (C) Closed level of channel, and (O) the direction of channel opening. This recording is representative of five independent experiments.

Discussion

Previous studies have used ^{36}Cl to track chloride movements in intact arteries. For example, norepinephrine (NE) stimulates ^{36}Cl efflux from rat aorta, femoral artery, and portal vein (14, 32–33). The peak agonist-stimulated increase in Cl^- permeability in response to NE (32) has been estimated to be two times greater than that seen for peak K^+ efflux. However, ^{36}Cl can be transported by multiple types of anion carriers (i.e., $Na^+/K^+/Cl^-$ cotransporter, Na^+-dependent Cl^-/HCO_3^- exchanger, and Na^+ independent Cl^-/HCO_3^- exchanger). Therefore, to analyze in greater detail the role of Ca^{2+} in mediating agonist-stimulated Cl^- movements via conductive channels, we utilized a combination of ^{125}I efflux measurements and patch-clamp techniques in cultured VSMCs. The results of the present studies clearly show that rat aortic VSMCs contain conductive Cl^- channels which are activated in response to vasoconstrictor agonists, such as Ang II and ATP. These Cl^- channels are regulated by $[Ca^{2+}]_i$, as shown by our observations that agonist-stimulated increases in ^{125}I efflux from VSMCs were mimicked using pharmacological agents (thapsigargin, ionomycin) which produce increases in

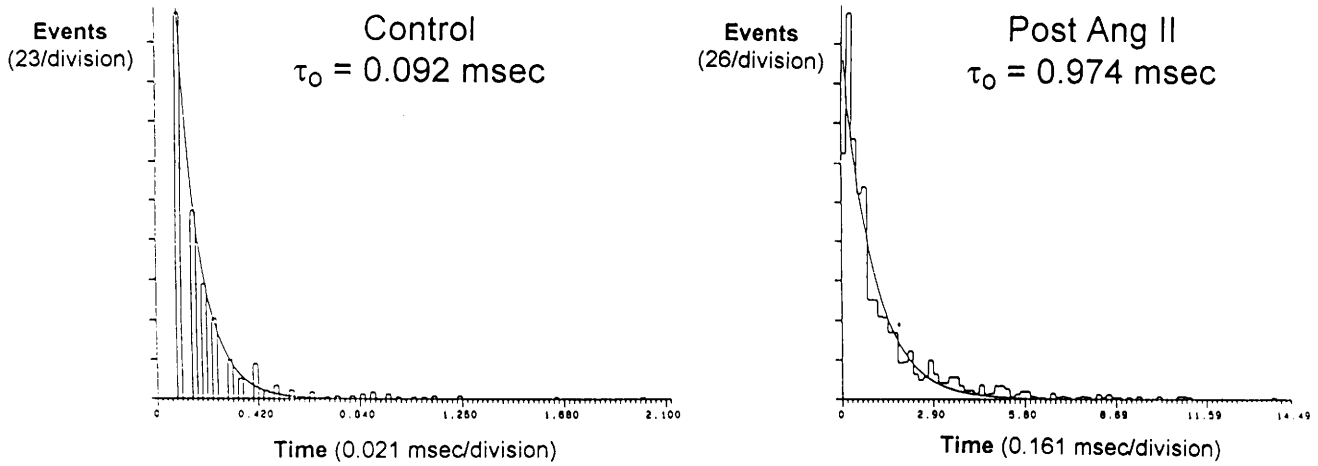

Figure 6. Chloride channel open time histograms prior to and following administration of Ang II (10^{-7} *M*) in a cell attached patch. In the left panel (control), sampled data were collected in 100 bins comprising 550 events, while in the right panel (+ Ang II), data were assigned to 88 bins composed of 1535 events. An open time constant (τ_o) was obtained under both experimental conditions by fitting a single exponential curve to each histogram. As shown in the figure, Ang II produced approximately a 10-fold increase in τ_o.

$[Ca^{2+}]_i$ and were inhibited using BAPTA-AM to dampen agonist-stimulated $[Ca^{2+}]_i$ increases. Furthermore, we found using patch-clamp techniques that Ang II stimulated a 75-pS Cl^- conductance in the C/A recording configuration which had a similar time course of activation as compared with Ang II-stimulated ^{125}I efflux in whole cells. Anion substitution experiments performed in excised patches have shown that the Ca^{2+}-sensitive Cl^- channel is relatively nonselective for halides (unpublished observation). Channel permeabilities were of the following rank order: iodide > bromide = fluoride > chloride. Presumably, under physiological conditions, the channel primarily conducts chloride ions.

Although the molecular mechanisms underlying Cl^- channel activation by vasoconstrictors in VSMCs have not been clearly defined, our data, as well as that of others (6–7, 25) may provide a cellular mechanism to explain previous observations that vasoconstrictors alter Cl^- homeostasis in blood vessels. Smith and Jones (32) have shown in intact arteries that the biphasic increase in Cl^- efflux in response to NE is dependent on internal Ca^{2+}. Approximately 50% of the early response (<1 min) remained after removing external Ca^{2+}, while the tonic response at 4.5 min was totally abolished by zero-Ca^{2+} solution. In the current study, BAPTA-AM loading of VSMCs inhibited Ang II-mediated ^{125}I efflux, thus providing strong evidence that agonist-stimulated ^{125}I efflux was activated by increases in $[Ca^{2+}]_i$. Although EGTA decreased basal ^{125}I efflux, it had no effect on Ang II-mediated increases in ^{125}I efflux, suggesting that Ca^{2+} release from intracellular stores was sufficient to initiate Cl^- channel activation. In freshly isolated porcine aortic VSMCs, Droogmans *et al.* (6) have shown that ATP increases $[Ca^{2+}]_i$ and stimulates an inwardly directed Cl^- selective current. Similar to our results, agonist-stimulated Cl^- channel activation depended on Ca^{2+} release from intracellular storage sites. Endothelin also depolarizes porcine coronary and human mesenteric VSMCs via an inward, Ca^{2+}-activated Cl^- current (7). This endothelin-induced I_{Cl} could be inhibited using BAPTA-AM to dampen, or mimicked using caffeine to induce, intracellular Ca^{2+} release in the whole-cell recording configuration.

It is well documented that Ca^{2+}-mobilizing agonists such as Ang II, endothelin, ATP, and NE stimulate phospholipase C-mediated phosphatidylinositol 4,5-bisphosphate in VSMCs yielding 1,4,5-inositol trisphosphate (1,4,5-IP_3) (34). 1,4,5-IP_3 stimulates Ca^{2+} release from the endoplasmic reticulum and has been shown to induce Ca^{2+}-activated Cl^- currents in various cell types (20–21). Microinjection of 1,4,5-IP_3 into VSMCs results in a decrease in Cl^- concentration as measured with the Cl^--sensitive fluorescent dye SPQ (25). The change in $[Cl^-]_i$ was linked to the activation of a Cl^- channel and an increase in membrane depolarization. Furthermore, it has been shown that intracellular dialysis of single human mesenteric VSMCs with heparin, a blocker of IP_3-induced Ca^{2+} release (34), inhibits endothelin-stimulated Cl^- currents (7). In contrast, heparin was ineffective in blocking Cl^- currents in response to caffeine which induces Ca^{2+} release from IP_3-insensitive Ca^{2+} pools (36). Interestingly, Takenaka *et al.* (26) found that the Cl^- channel blocker indanyloxyacetic acid has little effect on resting or peak $[Ca^{2+}]$ in endothelin-treated VSMCs but markedly attenuates the sustained phase of the Ca^{2+} transient. Furthermore, endothelin-induced contraction of rat aortic rings, as well as vasoconstriction of the rat renal microcirculation, can be inhibited by indanyloxyacetic acid (25–26). Collec-

tively, these data demonstrate that increased Cl^- channel activity may play an important role in regulating the sustained phase of VSMC contraction, perhaps by regulating Ca^{2+} influx pathways.

To date, the study of chloride channels in VSMCs has focused primarily on macroscopic or ensemble current activity recorded in the whole-cell recording configuration. Less information is available regarding single-channel current activity. Klockner recently reported the presence of Ca^{2+}-sensitive Cl^- currents in VSMCs isolated from human mesenteric artery (9). The calculated conductance of this channel was 2.8 ± 0.5 pS, a value somewhat lower than that which we report in rat VSMCs stimulated with Ang II (75 pS) in the current studies. This discrepancy may perhaps be explained by interspecies differences. Alternatively, VSMCs used in these studies were isolated from arteries of elderly surgical patients and the tissue displayed an elevated cholesterol content (9). It is uncertain whether the properties of this channel are representative of normal, human vascular tissue or whether they reflect an alteration in function associated with hypercholesterolemia and/or aging. Using an embryonic rat VSMC line, Soejima and Kokubun have characterized single-channel Cl^- currents which exhibit a conductance of 340 pS (8). This channel was quiescent in intact cells, however, current activity was observed in excised membrane patches. This result suggested that the channel was under the regulatory control of a cytoplasmic component. Channel function was Ca^{2+} independent, however, since current activity was insensitive to changes in bath $[Ca^{2+}]$. Subsequent studies showed that the Cl^- channel was activated in intact cells by protein kinase C (PKC) inhibitors (37). In the C/A recording configuration, inhibition of PKC permitted the expression of a large conductance channel which demonstrated similar characteristics to those currents seen in excised patches (37). The conductance of this channel and its pharmacological sensitivity is in contrast to the agonist-stimulated channel activity which we report in the current study. It has been shown that multiple chloride conductances may exist within a given cell type (e.g., cardiac myocytes [18] and epithelial cells [16]) and that these different currents may subserve diverse cellular functions. Results of the aforementioned studies suggest that multiple Cl^- channel types may exist in rat aortic VSMCs.

In summary, we suggest that Ca^{2+}-sensitive Cl^- channels ($I_{Cl(Ca)}$) may play an important role in mediating vascular contractile processes. Membrane receptor binding in response to a variety of agonists induces a rapid increase in $[Ca^{2+}]_i$ leading to the activation of the Cl^- channel. The resulting Cl^- efflux removes negative charge from the inside of the VSMC membrane, thus transiently depolarizing the cell membrane. A similar activation mechanism has been reported for phorbol ester-stimulated membrane depolarization of human neutrophils, where increases in Cl^- efflux were shown to selectively enhance membrane excitability (23). Membrane depolarization is a stimulus for the opening of voltage-sensitive Ca^{2+} channels which facilitate Ca^{2+} influx and the activation of contractile proteins in myocytes. $I_{Cl(Ca)}$-induced Ca^{2+} channel activation in response to agonists such as ATP, Ang II, and endothelin may therefore represent a common mechanism underlying vascular smooth muscle excitation.

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