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Inhibition by Clonidine of the Carbachol-Induced Tension Development and Nonselective Cationic Current in Guinea Pig Ileal Myocytes

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ABSTRACT—Effects of clonidine, an imidazoline derivative as well as α_2-adrenoceptor agonist, on carbachol (CCh)-evoked contraction in guinea pig ileal smooth muscle were studied using isometric tension recording. To investigate the cellular mechanisms of the inhibitory effect of clonidine, its effects on CCh-evoked nonselective cationic current (I_{CCh}), voltage-dependent Ca^{2+} current (I_{Ca}) and voltage-dependent K^+ current (I_K) was also studied using patch-clamp recording techniques in single ileal cells. Clonidine inhibited the contraction evoked by CCh (1 μM) in a concentration-dependent manner with an IC_{50} valve of 61.7 ± 2.5 μM. High K^+ (40 mM)-evoked contraction was only slightly inhibited even when clonidine was used at 300 μM. Externally applied clonidine inhibited I_{CCh} dose-dependently with an IC_{50} of 42.0 ± 2.6 μM. When applied internally via patch pipettes, clonidine was without effect. An I_{CCh}-like current induced by GTPγS was also inhibited by bath application of clonidine. None of KU14R and BU224, both imidazoline receptor blockers, and yohimbine, an α_2-adrenergic blocker, significantly affects the inhibitory effect of clonidine on I_{CCh}. Clonidine (300 μM) only slightly decreased membrane currents flowing through voltage-gated Ca^{2+} channels or K^+ channels. These data indicate that clonidine relaxes smooth muscle contraction produced by muscarinic receptor activation and suggest that the effect of clonidine seems due mainly to inhibition of I_{CCh} via acting directly on the involved cationic channel.

Keywords: Gastrointestinal smooth muscle, Carbachol, Cationic current, Clonidine, Imidazoline

Clonidine has been known as a centrally acting α_2-adrenergic receptor agonist that is used in clinical practice for its antihypertensive effect (1). However, its practical use for treatment of hypertension was limited because of its unpleasant side effects such as sedation and dry mouth. Today there is resurgence of interest in centrally acting agents because clonidine have repeatedly been shown to bind to distinct nonadrenergic sites with nanomolar affinities (2, 3) and its antihypertensive effect was mediated by a novel receptor type specific for imidazolines and its side effect was mediated by α_2-adrenergic receptor. These sites are classified into the imidazoline I_1 and the imidazoline I_2 sites; the I_1 sites are associated with control blood pressure and exhibit high affinity for clonidine and its derivatives (1, 4). Imidazoline I_2 receptors show a higher affinity for idazoxan and related imidazolines and are involved in eating behavior and psychiatric disorders such as major depression (5, 6). Meanwhile, others reported that some atypical imidazoline receptors, whose pharmacologic properties do not correspond to any of the foregoing subtypes, are involved in insulin secretion and were recently called imidazoline I_3 receptors (7), and another type of not-yet-classified imidazoline binding site with quite different pharmacological characteristics is present in various tissues (8), but its physiological roles are far less known.

Binding experiments with [^{3}H]clonidine and [^{3}H]idazoxan in membranes from guinea pig (9), rabbit (10) and rat gastric tissues (11) provided evidence suggesting that nonadrenergic binding sites for imidazolines are also present in the gastrointestinal tissue. Felsen et al. (12) showed that the clonidine-displacing substance (CDS), an endogenous ligand acting at the imidazoline binding sites, induced a concentration-dependent contraction of rat gastric fundus strips, but another group reported that these sites are neither directly related to a postsynaptic contractile effect on rat gastric smooth muscle nor to acid release from isolated gastric glands (13). Thus, although the effect of imidazoline derivatives on gastrointestinal smooth muscle contractility is still controversial, it is possible that the inhibitory effect of clonidine on intestinal motility is mediated by imidazoline binding sites and not by α_2-

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adrenceptors. Recent studies have shown that imidazoline derivatives block ATP-sensitive K^+ channels to stimulate insulin release in pancreatic β-cells and rat insulinoma (RIN) cells (14, 15). Imidazoline derivatives also inhibit the acetylcholine (ACh)-induced secretion of catecholamines in adrenal chromaffin cells (16) by blocking nicotinic ACh receptors (17). Finally, these compounds also interact with 5-HT_3 receptors in NIE-115 cells, inhibiting the veratridine-induced influx of guanidinium (11). Thus the mechanism of the imidazoline effect may be mediated by modulating various ion channels. In mammalian gastrointestinal myocytes, stimulation of muscarinic receptors by ACh or carbachol (CCh) induces nonselective cation current (I_{CCh}) (18). This current acts to depolarize the cell membrane, causing Ca^{2+} influx via voltage-gated Ca^{2+} channels. Thus I_{CCh} plays a role in the muscarinic receptor-mediated contraction of intestinal smooth muscle. Under the circumstances, it is possible that clonidine, an imidazoline derivative, may influence the intestinal contractility by modifying the activity of various channels including the muscarinic receptor cationic channel, voltage-dependent calcium channel and voltage-dependent potassium channel.

The present study was therefore, designed (1) to assess the effect of clonidine, the prototypical imidazoline derivative, on high K^+ solution-induced and CCh-induced contractions, and (2) to examine its effect on I_{CCh}, voltage-dependent Ca^{2+} current (I_{Ca}) and voltage-dependent K^+ current (I_K).

MATERIALS AND METHODS

Measurement of muscle tension

Guinea pigs of either sex, weighing 300 – 350 g, were exsanguinated after stunning. The ileum was isolated and cut into segments of 3- to 4-cm lengths and placed in physiological salt solution (PSS). The longitudnal muscle layer of intestinal segments was peeled from the underlying circular muscle and washed in PSS. Each ileal logitudinal muscle strip was attached to a glass holder and mounted vertically in a 20-ml organ bath filled with Krebs-Henseleit (K-H) solution. K-H solution was saturated with a mixture of 95% O_2 and 5% CO_2 at 37°C to maintain pH at 7.4. Muscle strips, loaded with a resting tension of 0.5 g, were equilibrated for more than 60 min before starting the experiment. Changes in contractile tension were measured isometrically with a force-displacement transducer and recorded on a pen-recorder. The muscle strip was repeatedly stimulated by isotonic 40 mM KCl until the response became stable and then the experiment was started.

Cell isolation

The longitudinal muscle layer of ileum was cut into small pieces and placed in Ca^{2+}-free PSS. The Ca^{2+}-free PSS was then replaced with PSS containing 30 μM Ca^{2+} (low Ca^{2+} PSS); and 30-min incubations at 37°C were carried out in fresh low Ca^{2+} PSS containing collagenase (0.3 mg/ml), papain (0.6 mg/ml), and bovine serum albumin (1 mg/ml). After this enzyme digestion, tissue fragments were suspended in a fresh 120 μM Ca^{2+} containing PSS and gently agitated. The resulting suspension was centrifuged at $600 \times g$ for 2 min, and the cells were resuspended in a 0.5 mM Ca^{2+} containing PSS, aliquoted into 12 mm poly-L-lysine-coated cover glasses and stored in a humidified atmosphere at 4°C. Experiments were carried out within 12 h of harvesting (22°C – 24°C).

Whole-cell voltage clamp

Whole-cell membrane currents were recorded at room temperature using standard patch-clamp techniques. The patch pipette had a resistance of 3 – 6MΩ when filled with a pipette solution. Membrane currents were measured with an Axoclamp 200A voltage-clamp amplifier (Axon Instrument, Union City, CA, USA). Command pulses were applied using an IBM-compatible computer and pCLAMP (version 6.0) software. The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder.

Solutions

The K-H solution was of the following composition: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 0.01 mM ethylenediamine tetraacetic acid (EDTA) and 5.5 mM glucose. Substituting NaCl with equimolar KCl in K-H solution made isotonic high K^+ (40 mM) solutions. The PSS used for cell isolation and the bath solution for potassium current recording had the following composition: 126 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, 10.5 mM *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethansulphonic acid] (HEPES) (titrated to pH 7.4 with NaOH). The patch pipette solution for the K^+ current recording had the following composition: 134 mM KCl, 1.2 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 0.05 mM ethylene glycol-bis(β-aminoethylether)-*N,N,N'N'*-tetraacetic acid (EGTA), 14 mM glucose and 10.5 mM HEPES (titrated to pH 7.2 with NaOH). The bath solution for recording the barium current through calcium channels had the following composition: 126 mM NaCl, 6 mM TEACl, 2 mM $BaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, 10.5 mM HEPES (titrated to pH 7.2 with NaOH). The pipette solution for recording the barium current through calcium channels had the following composition: 134 mM CsCl, 1.2 mM $MgCl_2$, 1 mM MgATP, 0.1 mM Na_2GTP, 0.05 mM EGTA, 14 mM glucose and 10.5 mM HEPES (titrated to pH 7.2 with CsOH). The bath solution for recording I_{CCh} had the following composition: 120 mM CsCl, 12 mM glucose, 10 mM HEPES (titrated to pH 7.4

with CsOH) (total $Cs^+ = 124$ mM). The pipette solution for recording I_{CCh} had the following composition: 80 mM CsCl, 1 mM MgATP, 5 mM creatine, 1 mM Na_2GTP, 10 mM BAPTA, 20 mM glucose and 10 mM HEPES, 4.6 mM $CaCl_2$ (calculated $[Ca^{2+}]_i = 100$ nM (titrated to pH 7.2 with CsOH) (total $Cs^+ = 124$ mM). The presence of 1 mM GTP in this solution reduced desensitization to a minimum (19). In experiments designed to activate cationic channels, without muscarinic receptor activation, GTP in the pipette solution was replaced with 200 μM GTPγS (19, 20).

Solution application

Test solutions were applied to single ileal cells via a gravity-fed fused silica capillary tube connected to an array of six polyethylene tubes. The outlet of the perfusion system was located within 100 mm of the cell. Drug application was started by switching the control external solution to a drug solution. Complete solution exchange occurred within 1< s.

Data analyses

Concentration-effect curves were fitted by a logistic function in the following form:

$$\frac{I_{clonidine}}{I_{control}} = \frac{1}{1+([clonidine]/IC_{50})^p}$$

where $I_{control}$ and $I_{clonidine}$ are the cationic current amplitudes in the absence and presence of various clonidine concentrations, [clonidine], respectively; IC_{50} equals the clonidine concentration at which current amplitude was reduced by 50% and p is the slope factor of the inhibition curve. The data was analyzed and plotted using GraphPad Prism software version 3.0 (San Diego, CA, USA). Values are given as the means ± S.E.M.

Drugs

The following drugs and chemicals were used: Collagenase (type 1A), adenosine 5'-triphosphate (ATP, magnesium salt), guanosine 5'-triphosphate (GTP, sodium salt), clonidine hydrochloride, carbamylcholine chloride (carbachol), yohimbine, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS, tetralithium salt), creatine, HEPES, 1,2-bis(2-aminophenoxy) ethane-*N,N,N'N'*-tetraacetic acid (BAPTA), EGTA were obtained from Sigma Chemical, (St. Louis, MO, USA). KU14R (2-(2-ethyl-2,3-dihydro-2-benzofuranyl)-1*H*-imidazole and BU224 (2-(4,5-dihydroimidazol-2-yl)quinoline) were from Tocris (Bristol, UK).

RESULTS

Changes in muscle force

Figure 1 shows the inhibitory effect of cumulative application of clonidine on the sustained contraction induced by high K^+ (40 mM) solution and CCh (1 μM) in guinea pig ileal segment. The CCh-induced contraction was more sensitive to clonidine than the high K^+-induced contraction. IC_{50} of clonidine on the carbchol-induced contraction was 61.7 ± 2.5 μM (n = 8). Clonidine decreased the high K^+-induced contraction only by some 20% even when applied at 300 μM.

Carbachol-activated current (I_{CCh}) in ileal myocytes

In cells voltage-clamped at −40 mV, inward cationic current was evoked by bath application of CCh after at least 3 min elasped from break-through of the patch membrane. CCh was applied at 50 μM, a concentration close to the maximally effective one in these cells (21). The current-voltage (I-V) relationship was measured with a negative going ramp pulse from 80 mV to −120 mV over 6 s (Fig. 2A, control trace). The I-V relationship of I_{CCh}

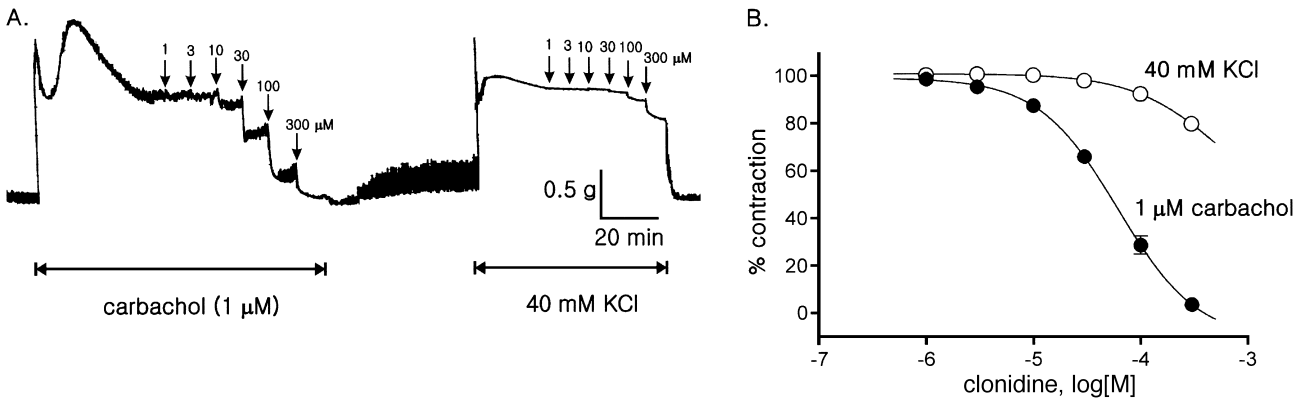

Fig. 1. Effects of cumulative application of clonidine on muscle force of guinea pig ileal longitudinal muscle. A) Typical tracing of effects of clonidine on muscle force evoked with 1 μM carbachol and 40 mM KCl. B) Concentration-response curves of clonidne effect on muscle force of guinea pig ileum. 100% represents the muscle force induced by 1 μM carbachol and 40 mM KCl before the cumulative addition of clonidine.

showed inward rectification at around +15 mV and a negative slope conductance at negative potential range (<40 mV, Fig. 2A, control trace); that is, it has a typical U-shaped dependence on the membrane potential. All these properties are similar to those of the muscarinic receptor-operated nonselective cationic channels (21, 22).

Effect of clonidine on I_{CCh}

I_{CCh} evoked by 50 μM CCh was suppressed by clonidine externally and cumulatively applied at ascending concentrations of 10 – 300 μM (Fig. 2A). The current suppression increased with increasing clonidine concentration and reversed nearly completely within about 1 min when clonidine was washed away. The dose-dependency of the inhibitory effect of clonidine is shown in Fig. 2B. The degree of inhibition was estimated as the ratio of remaining current to the control current. A mean value of IC_{50} was 42.0 ± 2.6 μM (n = 4).

Figure 3A shows a representative of experiments designed to see if clonidine can act from the inside of the

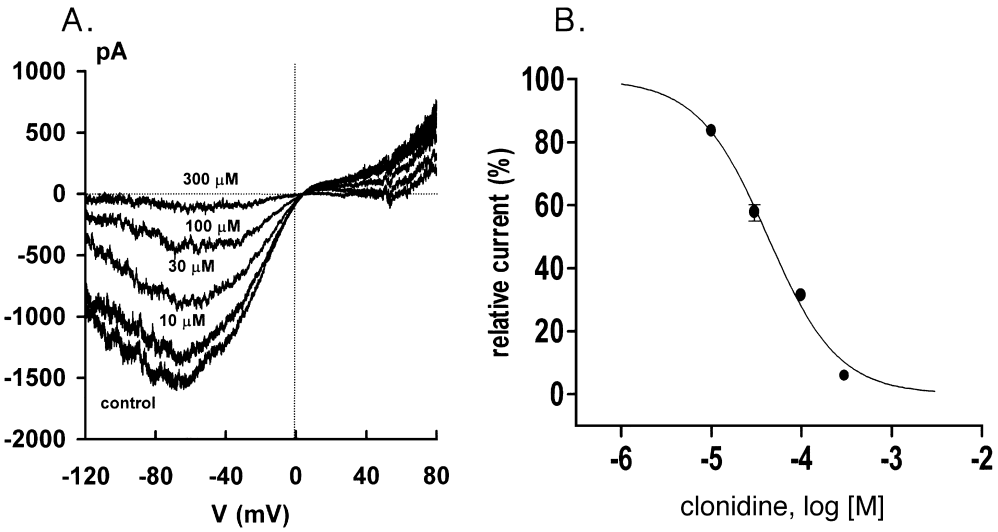

Fig. 2. Effects of clonidine on carbachol-activated cationic current (I_{CCh}) in single guinea pig ileal smooth muscle cells. A) Steady-state current-voltage relationships measured by 6-s duration voltage ramps in the presence of 50 μM carbachol and clonidine at the indicated concentration. B) Concentration-response curve of clonidine on I_{CCh}.

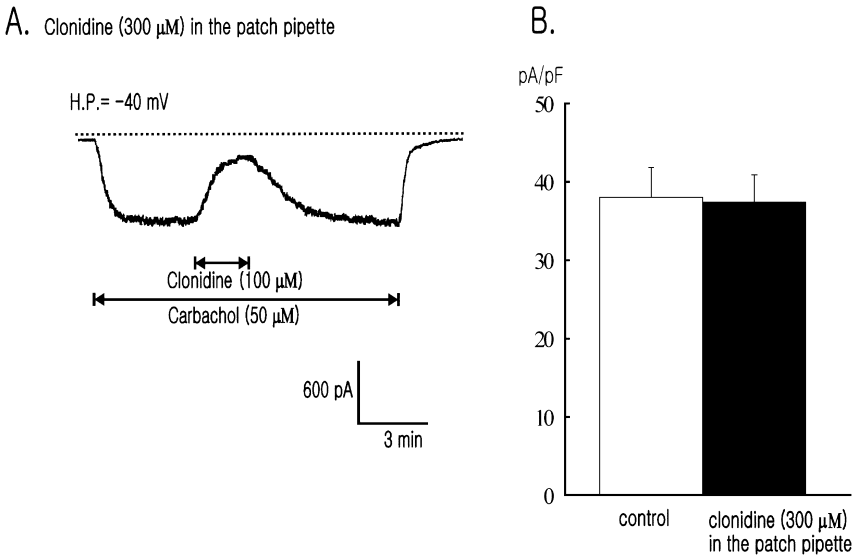

Fig. 3. Effects of clonidine in the pipet solution on I_{CCh} in single guinea pig ileal smooth muscle cells. Clonidine was added at 300 μM, the maximally suppressing dose when externally applied, in the pipette solution. A) Typical tracing of effects of clonidine on I_{CCh} in single guinea pig ileal smooth muscle cells in the presence of clonidine (300 μM) in the pipette solution. B) Comparison of amplitude of I_{CCh} between the absence and presence of clonidine (300 μM) in the pipette solution. Inward current induced by 50 μM carbachol were normalized to the membrane capacitance measured (mean ± S.E.M., n = 4).

cell to block the I_{CCh}. Clonidine (300 μM) was applied internally via patch pipettes. I_{CCh} amplitudes, expressed per the membrane capacitance, were 38.0 ± 3.8 pA/pF (n = 4) in the absence and 37.8 ± 3.5 pA/pF (n = 4) in the presence of intracellular clonidine. There was no significant difference between the mean values (Fig. 3B). When clonidine was externally applied during generation of I_{CCh} in cells internally treated with clonidine, the I_{CCh} was profoundly inhibited (Fig. 3A). Therefore, clonidine seems likely to act only from the outside of the cell to block I_{CCh}.

Voltage dependence of the clonidine effect on I_{CCh}

Steady-state I-V relationships in the presence of 50 μM CCh and clonidine (100 μM) were measured using the same ramp pulse as described in Fig. 2. I_{CCh} was partially suppressed by 100 μM of clonidine. The ratio of I_{CCh} amplitude in the presence of clonidine to that in its absence was plotted against membrane potential (n = 3) (Fig. 4). The degree of I_{CCh} inhibition by the clonidine decreased with membrane depolarization; the degree of I_{CCh} inhibition at −120 mV was 2.1 ± 0.3 (n = 3) times greater than that at −10 mV.

Influence of adrenergic and imidazoline receptor antagonists on the inhibitory effect of clonidine

Clonidine (100 μM) suppressed I_{CCh} evoked at −40 mV to 68.5 ± 1.7% (n = 4). A 15-min pretreatment with yohimbine (1 μM), an α_2-adrenoceptor antagonist, did not influence the inhibition by clonidine (66.3 ± 1.9% suppression, n = 4) (Fig. 5A). A substantially similar result was obtained with KU14R (10 μM), an imidazoline I_1-receptor antagonist (68.2 ± 2.4% suppression, n = 4) (Fig. 5B) or BU224 (10 μM), an imidazoline I_2-receptor antagonist (62.1 ± 2.1% suppression, n = 5) (Fig. 5C).

These suggest that clonidine may cause I_{CCh} inhibition by acting on some sites other than imidazoline I_1 receptors, I_2 receptors and α_2-adrenoceptors.

The effect of clonidine on GTPγS-activated current

It is known that muscarinic stimulation activates non-selective cation channels through the activation of GTP-binding protein-mediated signal transduction (22). As shown in Fig. 6B, in cells held at −40 mV, intracellular application of 200 μM GTPγS via patch pipettes produced inward cationic current, which developed slowly to reach a steady-state level within 3 – 5 min and then sustained for several 10 min as previously reported (19). The GTPγS-activated

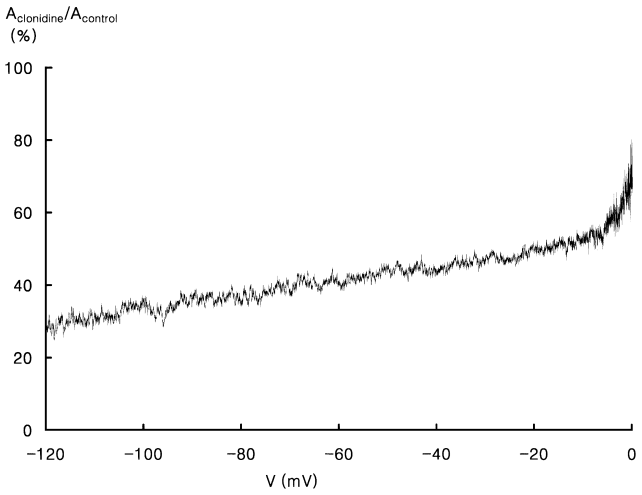

Fig. 4. Voltage-dependency of the effect of clonidine on I_{CCh}. Stimulation protocol is the same with Fig. 2. Steady-state current-voltage relationships measured by 6-s duration voltage ramps in the presence of 50 μM carbachol and clonidine (100 μM). I_{CCh} was partially suppressed by 100 μM of clonidine. The ratios of amplitude of I_{CCh} applied with clonidine to those of control currents were plotted against membrane potential (n = 3).

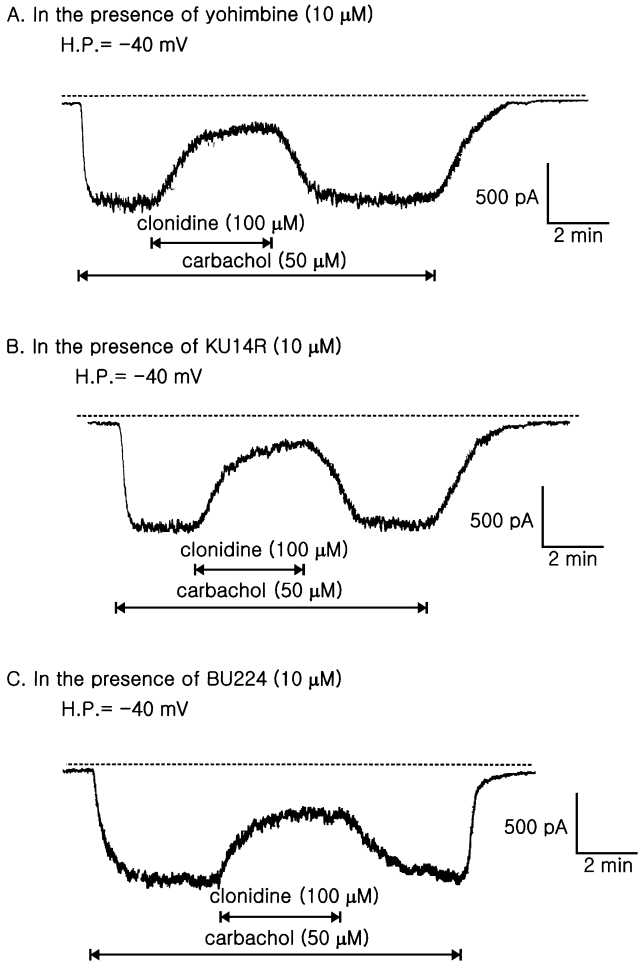

Fig. 5. Yohimbine (A), KU14R (B) and BU224 (C) effects on the suppressive effect of clonidine on I_{CCh}. The perparations were pretreated with yohimbine, KU14R or BU224 for 15 min, and I_{CCh} was recorded at −40-mV holding potential. All of the antagonists did not block the clonidine effect on I_{CCh}.

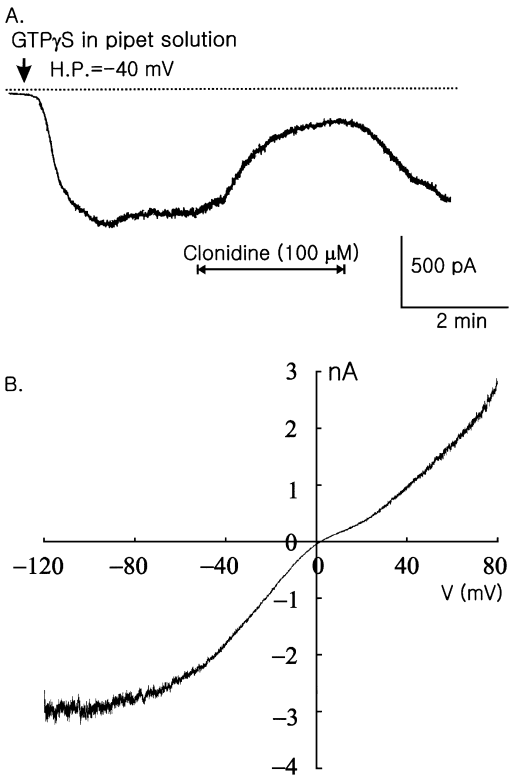

Fig. 6. Effects of clonidine on GTPγS-induced current. A) GTPγS (0.2 mM) was added in the pipette solution. Slowly increasing inward current was induced 2 min after break through (arrowhead). B) This current-voltage (I-V) relation curve was recorded with the same method as Fig. 2. This I-V curve are similar to those induced by carbachol (see Fig. 2, control trace).

current showed time- and voltage-dependent characteristics similar to those of I_{CCh} (Fig. 6B), and it was also inhibited by 100 μM clonidine (72.1 ± 1.1% suppression, n = 4) (Fig. 6A). Therefore, it seems likely that the binding site for clonidine is the cationic channel rather than the muscarinic receptor.

Effect of clonidine on voltage-dependent Ca^{2+} channels and K^+ channels

The effects of clonidine on barium current (I_{Ba}) through voltage-dependent Ca^{2+} channels were investigated. I_{Ba} was evoked by stepping to various depolarizing levels from a holding potential of −60 mV. As shown in Fig. 7A, clonidine (300 μM) produced only a small decrease in the amplitude of I_{Ba}; the percent inhibition of the current activated at 0 mV was 11.6 ± 2.6% (n = 4) on average.

As shown in Fig. 7B, clonidine (300 μM) also slightly suppressed I_K evoked by stepping to various potentials (up to 80 mV) from −60 mV. Actually, the percent inhibition of I_K evoked at 30 mV was 14.9 ± 4.4% (n = 4).

DISCUSSION

The actions of the imidazoline derivative clonidine in the guinea pig ileal longitudinal smooth muscle can be summarized as follows: 1) it inhibited CCh-evoked contraction in a dose-dependent manner, 2) the inhibition seemed to be brought about by suppression of I_{CCh}.

As mentioned in the introduction, it is reported that imidazoline receptors are located in various tissues and play a role according to their physiological circumstances. For example, the imidazoline receptors have been identified in several kinds of smooth muscle cells (23, 24) and suggested to contribute to regulation of muscle tone. In the gastrointestinal tract, radioligand binding data provide evidence that the rat and human stomachs are endowed with I_2-like binding sites and non-I_1/non-I_2-[^{3}H]clonidine binding sites that represent two different entities, suggesting that they participate in the regulation of intestinal smooth muscle motility (16).

In the present study, clonidine suppressed CCh-evoked contraction with the IC_{50} value of 61.7 μM range. The K_d of clonidine for α_2-adrenoceptors is estimated to be in the nanomolar range (7.3 nM) in vascular smooth muscle (25). These, therefore, suggested that the action of clonidine is not mediated by α_2-adrenoceptors.

As mentioned in the introduction, imidazolines have been suggested to block various ion channels including the nicotonic acetycholine receptor channel (17), the $5HT_3$ receptor channel (26), ATP-sensitive K^+ channel (14) and the NMDA channel (27) with IC_{50} of 10 micromolar range possibly by binding to a phencyclidine recognition site within their ion-conducting pore. Inhibition of single-channel conductance in cell-free patches by I_1-receptor ligands is highly suggestive of direct blockade (28). Furthermore, imidazolines displace [^{3}H]phencyclidine and [^{3}H]MK801 from nicotinic ACh receptors and NMDA receptors, respectively, consistent with direct activation at the pore (17). Phentolamine, an imidazoline derivative, can block currents flowing via ATP-sensitive K^+ channel expressed in *Xenopus* oocytes (29), also indicating the direct action of imidazolines at the pore of this channel. In the gastrointestinal tract, the signal transduction pathway between muscarinic receptor activation and ion channels has been studied in guinea pig ileal myocytes (22). The muscarinic agonist ACh causes membrane deporlarization of smooth muscle and thus an increase in Ca^{2+} influx via voltage-gated Ca^{2+} channels. The depolarizing action is mediated primarily by M_2 muscarinic receptor activation (21, 30) which is linked to the voltage-dependent, $[Ca^{2+}]_i$-sensitive cationic channels via G_i/G_o proteins (30). In the present study, we found for the first time, that clonidine suppressed the I_{CCh} with the IC_{50} value of 42 μM, similar to the corresponding values reported for various other

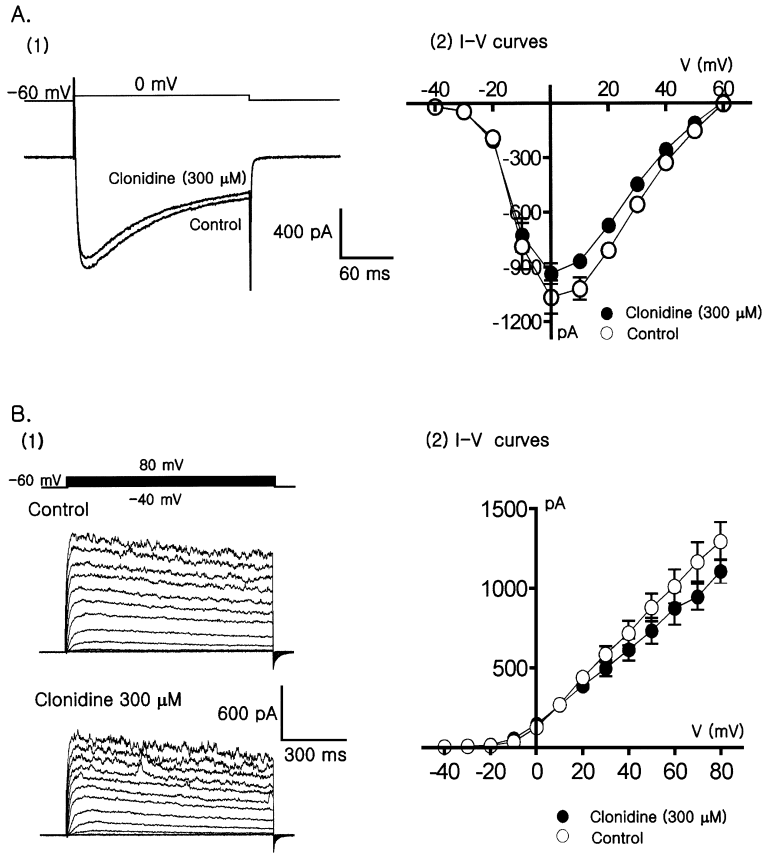

Fig. 7. Effects of clonidine on voltage activated Ba^{2+} current through Ca^{2+} channel and K^+ currents in single ileal smooth cells of guinea pig. A, 1) Ba^{2+} current was elicited by 200-ms depolarization from −60 mV to 0 mV at 0.1 Hz. Clonidine, 300 μM, the maximally I_{CCh}-suppressing dose when applied externally, was applied in the bath solution. A, 2) Current-voltage relationships for the peak inward Ba^{2+} current activated from a holding potential of −60 mV. The peak amplitude of Ba^{2+} current evoked by stepping to different potential from holding potential is plotted against the potential. B, 1) In each panel, the cell was held at −60 mV, and test depolarization with duration 1 s were applied from −40 mV to +80 mV in 10-mV increments. Currents are recorded using a pipette containing 0.05 mM EGTA. Clonidine at 300 μM, the maximally I_{CCh}-suppressing dose when applied externally, was applied in the bath solution. B, 2) Current-voltage relationships for the outward K^+ current activated from a holding potential of −60 mV. The sustained amplitude of K^+ current evoked by stepping to different potential from the holding potential is plotted against the potential.

types of ion channels (see above). This IC_{50} was also well matched with that for clondine effect on CCh-induced contraction. Substances affecting neurotransmitter-operated channels like this CCh-activated channel may act at any of the steps between ligand-receptor binding and channel gating. However, indirect interference of clonidine with activation of the G-protein by the receptor is not likely because: 1) clonidine suppressed the GTPγS-activated current (Fig. 6) and 2) it failed to inhibit I_{CCh} when applied intracellularly (Fig. 3). 3) Furthermore, neither yohimbine (α_2-adrenoceptor antagonist) nor KU14R (imidazoline I_1 receptor antagonigst), blocked the clonidine effect on I_{CCh} (Fig. 5). So clonidine seems to act directly on the cation channel mainly from the external side of the membrane. This is contradictive to the previous report that imidazolines do not inhibit non-selective cation channels whose opening is mediated by G_i-protein-coupled α_{2A}-adrenoceptors in human erythroleukemia (HEL) cells (31). Therefore, imidazoline effect on the cationic channel might be tissue- and/or activated receptor-specific.

Could the clonidine-binding site on the muscasrinic receptor-operated cationic channel actually be the imidazoline receptor? At first sight, it would seem not, as clonidine action on I_{CCh} was blocked neither by KU14R, a putative imidazoline I_1-receptor antagonist nor by BU224, I_2-receptor antagonist, and the apparent affinities for clonidine for the I_{CCh} are several orders of magnitude lower than the affinity of these drugs for I_1 sites. However, radioligand binding experiments carried out in cell membrane from guinea pig small intestine also revealed the existence of another non-adrenergic [^{3}H]clonidine binding site, namely, non-I_1/non-I_2-[^{3}H]clonidine binding sites that are different

entities (13). The characteristics of these binding sites were not clarified. So, whether the clonidine-binding site responsible for I_{CCh} inhibition is an imidazoline receptor cannot be determined at present. Naturally, a connection between the clonidine binding site for I_{CCh} inhibition and the imidazoline receptor is purely speculative at this stage; to demonstrate this connection, further study needed.

Meanwhile, clonidine slightly suppressed the I_K (11.6% decrease at 30 mV) or I_{Ca} (14.9% decrease at 0 mV) in guinea pig ileal longitudinal smooth muscle at 300 μM. Needless to say, clonidine may inhibit the I_{Ca} more significantly at more than 300 μM. However, clonidine can inhibit I_{CCh} at much lower concentration ($IC_{50} = 42.0 \pm 2.6$ mM). Thus the inhibitory effect of clonidine on carbachol-induced contraction of guinea pig ileal longitudinal tract is mainly mediated by suppression of I_{CCh} through involved cation channels. These results are not inconsistent with the previous report that the voltage-operated calcium channel is only slightly affected by most imidazolines that are ligands for I_1 receptors (31).

The voltage-dependence of I_{CCh} inhibition by clonidine resembled that reported for quinidine, TEA, 4-AP, caffeine and quinine in ileal myocytes (32). The I_{CCh} inhibition by these agents was strongly attenuated by membrane depolarization. Such blockade tends to voltage-dependent because the interference would be relieved by depolarization due to the reduction of driving force.

In conclusion, clonidine relaxes the ileal smooth muscle pore contracted by CCh, possibly by the inhibiting I_{CCh}, a depolarzing current, via a direct blocking action on the muscarinic receptor-operated cationic channel. This action may underlie the regulating effect on intestinal motility by imidazolines observed so far.

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