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Inhibition of the calcium-activated chloride current in cardiac ventricular myocytes by *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA)

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ABSTRACT

N-(*p*-amylcinnamoyl)anthranilic acid (ACA), a phospholipase A_2 (PLA_2) inhibitor, is structurally-related to non-steroidal anti-inflammatory drugs (NSAIDs) of the fenamate group and may also modulate various ion channels. We used the whole-cell, patch-clamp technique at room temperature to investigate the effects of ACA on the Ca^{2+}-activated chloride current ($I_{Cl(Ca)}$) and other chloride currents in isolated pig cardiac ventricular myocytes. ACA reversibly inhibited $I_{Cl(Ca)}$ in a concentration-dependent manner (IC_{50} = 4.2 μM, n_{Hill} = 1.1), without affecting the L-type Ca^{2+} current. Unlike ACA, the non-selective PLA_2 inhibitor bromophenacyl bromide (BPB; 50 μM) had no effect on $I_{Cl(Ca)}$. In addition, the analgesic NSAID structurally-related to ACA, diclofenac (50 μM) also had no effect on $I_{Cl(Ca)}$, whereas the current in the same cells could be suppressed by chloride channel blockers flufenamic acid (FFA; 100 μM) or 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS;100 μM). Besides $I_{Cl(Ca)}$, ACA (50 μM) also suppressed the cAMP-activated chloride current, but to a lesser extent. It is proposed that the inhibitory effects of ACA on $I_{Cl(Ca)}$ are PLA_2-independent and that the drug may serve as a useful tool in understanding the nature and function of cardiac anion channels.

1. Introduction

Chloride channels that are activated by intracellular Ca^{2+} are present in various tissues where they are involved in different physiological functions [1]. In the heart, Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) have been identified in atria [2], ventricles [3–5] and Purkinje fibres [6] of different animal species. Functionally, the cardiac $I_{Cl(Ca)}$ participates in the early phase of repolarization during an action potential [3,7]. Under disease conditions, the transient inward component of $I_{Cl(Ca)}$ has been implicated in arrhythmogenesis [8,9].

Despite the importance of Ca^{2+}-activated chloride channels, progress in understanding their structure, function and regulation has been limited by the uncertainty about the underlying channel protein and a lack of specific antibodies (see [10] for review). As a result, pharmacological agents still provide the major tools to study these channels. The agents include structurally diverse chemicals such as 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), disulfonic stilbene derivatives like 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and the non-steroidal anti-inflammatory drugs (NSAIDs) of the fenamate group like flufenamic and niflumic acids. However, most of the drugs available to inhibit Ca^{2+}-activated Cl^- channels are only effective at high concentrations and are non-specific. For example, NPPB also blocks L-type Ca^{2+} currents [11], whereas niflumic acid not only inhibits but also partly stimulates $I_{Cl(Ca)}$ [12] and may cause release of Ca^{2+} from intracellular stores [13,14]. In addition, disulfonic stilbene derivatives are mostly effective at high concentrations and their effects are not readily reversible. Consequently, additional drugs that modulate $I_{Cl(Ca)}$ could be valuable in developing more specific inhibitors as well as in providing further insights into the functional role and nature of the channels. Among drugs structurally-related to fenamates is *N*-(*p*-amylcinnamoyl)anthranilic acid

Abbreviations: ACA, *N*-(*p*-amylcinnamoyl)anthranilic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; $I_{Cl(Ca)}$, calcium-activated chloride current; $I_{Cl(cAMP)}$, cyclic AMP-activated chloride current; I_{to}, transient outward current; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; NSAID, non-steroidal anti-inflammatory drug; PLA_2, phospholipase A_2; TRP, transient receptor potential.

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(ACA), also known to inhibit phospholipase A_2 (PLA_2; [15]). Here, we show that ACA inhibits $I_{Cl(Ca)}$ in a concentration-dependent manner without affecting L-type Ca^{2+} currents.

2. Materials and methods

2.1. Cell isolation and electrophysiology techniques

The study was approved by the Ethical Commission on Animal Experiments of the University of Leuven and carried out according to the institutional guidelines for laboratory animal care. We used ventricular myocytes dissociated from pig by methods described in detail before [16–18]. Briefly, pigs were anesthetized with sodium pentobarbitone (5–15 mg kg^{-1} I.V.), intubated and ventilated, and then injected with heparin (5–8 mg kg^{-1} I.V.) and a sodium pentobarbitone overdose (100 mg kg^{-1}) before tissue extraction. Cells were dissociated by enzymatic tissue digestion during Langendorff perfusion. All experiments were performed at room temperature. The voltage-clamp protocols consisted of either steps from the holding potential of −80 mV to various levels or 4-s symmetrical ramps from −120 to +80 mV and back to −120 mV, applied every 10 s. During the ascending limb of the ramp, the slow rate of depolarization (0.1 V/s) allowed activation and inactivation of the voltage-dependent Na^+ current. Currents were measured during the descending limb of the ramp. The pClamp 8.1 software was used to generate voltage protocols and to record data via a Digidata 1322A acquisition system (Axon instruments, Union City, CA, USA).

2.2. Solutions and drugs

The standard Tyrode solution used during cell dissociation contained (in mM): 135 NaCl, 5.4 KCl, 0.9 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10 HEPES and 10 glucose; pH was adjusted to 7.4 with NaOH. During voltage clamp measurements, cells were superfused with a solution of similar composition except that K^+ was replaced by Cs^+. When studying Ca^{2+}-activated Cl^- currents, Na^+-free solutions (with Na^+ replaced by $NMDG^+$) were used to eliminate the voltage-dependent Na^+ currents. For low Cl^- solutions, Cl^- was replaced by glutamate or by methylsulfate. The standard pipette solution contained (in mM): 130 Cs-glutamate, 25 TEA-Cl, 5.5 $MgCl_2$, 5 Na_2ATP, 1 EGTA, 0.1 Na_2GTP, 5 HEPES (pH 7.25; adjusted with CsOH) and was modified in a few experiments by replacing EGTA with BAPTA.

ACA was obtained from Tebu-Bio (Boechout, Belgium). All other drugs or chemicals were from Sigma–Aldrich (Bornem, Belgium) or Merck (Darmstadt, Germany). Nifedipine was prepared as stock solution in ethanol, whereas ACA, bromophenacyl bromide, DIDS, flufenamic acid and forskolin were prepared in DMSO. All other chemicals were dissolved in water.

2.3. Data and statistical analyses

Data were analyzed using Clampfit 8.2 (Axon Instruments) and Origin 7 (Microcal, USA). The following Hill equation was used to fit the inhibiting effects of ACA as a function of concentration:

$$\text{Relative current} = I_{[D]}/I_{max} = 1/[1 + ([D]/IC_{50})^{n_{Hill}}],$$

where $I_{[D]}$ is the current at a given drug concentration $[D]$, I_{max} is the current in the absence of the drug, IC_{50} is the drug concentration for 50% inhibition, and n_{Hill} is the Hill coefficient. Average data are expressed as mean ± standard error of the mean, with n indicating the number of cells studied. Means were compared using the two-tailed Student's t-test. $P \leqslant 0.05$ was taken as threshold for statistical significance.

3. Results

3.1. The Ca^{2+}-activated chloride current ($I_{Cl(Ca)}$) in pig cardiac ventricular myocytes

Fig. 1A illustrates typical whole-cell current traces obtained using voltage steps between −80 and +60 mV (see inset) in Na^+-free conditions. The steps induced capacitive transients, which (at potentials >−40 mV) were followed by short-lived biphasic currents with brief inward and prominent outward components (Fig. 1A, panel *a*; see also left panels of Figs. 2A and 3B and C). Both inward and outward currents could be completely suppressed by nifedipine (5–100 μM; $n = 15$; not illustrated), suggesting that the biphasic currents may be due to superimposed inward L-type Ca^{2+} currents ($I_{Ca,L}$) and a Ca^{2+}-activated outward current. We tested the hypothesis that the outward current was a Cl^- current ($I_{Cl(Ca)}$) by removing extracellular chloride (Cl_o^-) and by using Cl^- channels inhibitors. When Cl_o^- was replaced by glutamate, the currents induced by depolarization became monophasic inward with no outward component (Fig. 1A, panel *b*). This effect was reversible upon re-admission of Cl_o^- (Fig. 1A, panel *c*). In the presence of Cl_o^-, flufenamic acid (100 μM), a known Cl^- channel inhibitor, suppressed the outward transients, leaving inward currents resembling those in Cl^--free solutions (Fig. 1A, panel *d*; similar results in seven cells). DIDS (100 μM), another Cl^- channel inhibitor, also suppressed the outward transients, but to a lesser extent ($n = 4$; not illustrated). Fig. 1B shows that the Cl^--dependent current, calculated as the difference between the current in the presence of Cl_o^- and that after Cl_o^- removal, had a maximum value (1.46 ± 0.26 pA/pF; $n = 5$) at +25 mV. Furthermore, in five other cells dialyzed with 10 mM BAPTA-containing solution and treated with 10 μM ryanodine in the external solution to prevent sarcoplasmic Ca^{2+} release, only $I_{Ca,L}$ was observed (see Fig. 2B, left panel), demonstrating a dependence of the outward current on intracellular Ca^{2+}. Taken together, these results indicate that the transient outward current observed in our experiments is $I_{Cl(Ca)}$ and are consistent with those by others showing the presence of $I_{Cl(Ca)}$ in pig ventricular myocytes [19].

In contrast to the effect on the transient currents elicited at potentials >−40 mV, the removal of Cl_o^- did not affect the steady-state current in the same cells (Fig. 1C), indicating that the Cl_o^- removal was not altering background conductance.

3.2. Effects of N-(p-amylcinnamoyl)anthranilic acid (ACA)

We then tested the effect of ACA on $I_{Cl(Ca)}$. Fig. 2 shows currents obtained using depolarizing steps, in control conditions and in the presence of ACA. Upon application of ACA (20 μM) outward components were suppressed (Fig. 2A, middle panel), an effect that was reversible upon drug washout (not illustrated). The ACA-sensitive current at various potentials (calculated as the difference between control current and the current during ACA application) is shown in Fig. 2A (right panel). Its current–voltage relationship in five cells (Fig. 2C, open symbols) is similar to that of the Cl^--dependent current (see Fig. 1B). To exclude the possibility that the effect of ACA could be due to an enhancement of $I_{Ca,L}$, with a masking of a superimposed outward current, we tested the effect of ACA under conditions where intracellular Ca^{2+} release was inhibited with ryanodine and cytosolic Ca^{2+} was highly buffered with BAPTA. Outward transients were absent in BAPTA-dialyzed and ryanodine-treated cells (Fig. 2B, left panel), and the control current ($I_{Ca,L}$ uncontaminated by $I_{Cl(Ca)}$) was not affected by ACA (Fig. 2B, middle panels; 103 ± 7% of control at 0 mV; $P = 0.548$ compared with control; paired t-test, $n = 5$), i.e. the ACA-sensitive current was practically zero at all tested voltages (Fig. 2B, right panel, and C, filled

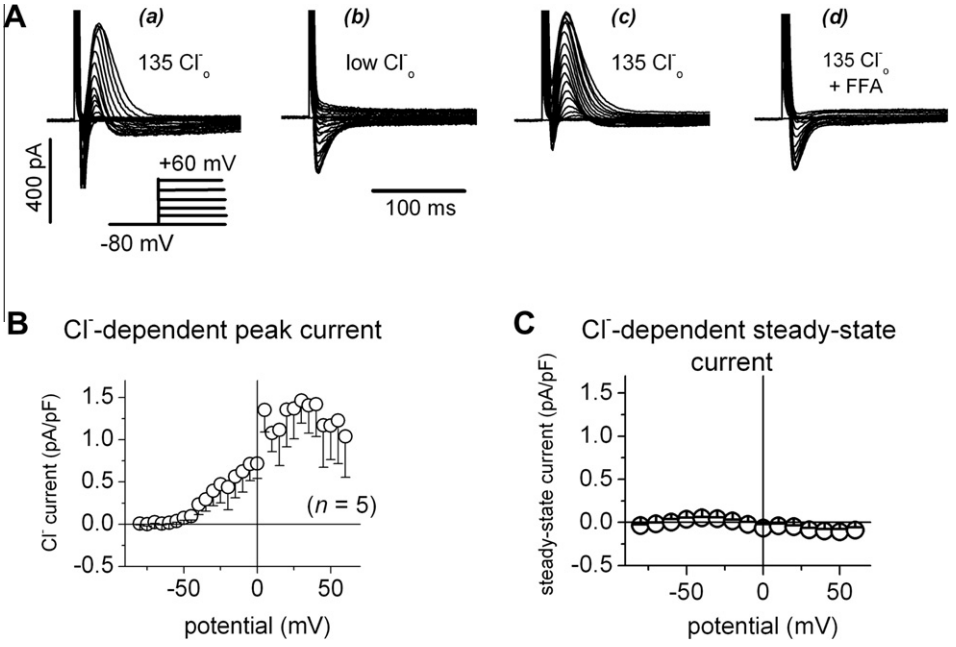

Fig. 1. Presence of a Ca^{2+}-activated Cl^- current in pig ventricular myocytes. (A) Traces of whole-cell currents induced by voltage steps from −80 mV to various levels (between −80 and +60 mV; see inset) in a pig ventricular myocyte dialyzed with the standard internal solution and superfused with Na^+-free, $NMDG^+$-containing solution. Panel *a*: typical currents in the presence of extracellular chloride (Cl^-_o): capacitive currents followed by inward and outward transient currents. Panel *b*: effect of removing Cl^-_o (Cl^- replaced by glutamate). Notice the absence of outward transient currents after the capacitive currents. Panel *c*: effect of re-admission of Cl^-_o. Panel *d*: effect of adding extracellular flufenamic acid (FFA; 100 μM) in the presence of Cl^-_o. (B and C) Voltage-dependence of peak (B) or steady-state (C) Cl^-_o-sensitive current. The Cl^-_o-sensitive current was calculated as difference between the currents in the presence and after the removal of Cl^-_o. The end-of-pulse current was taken as steady-state.

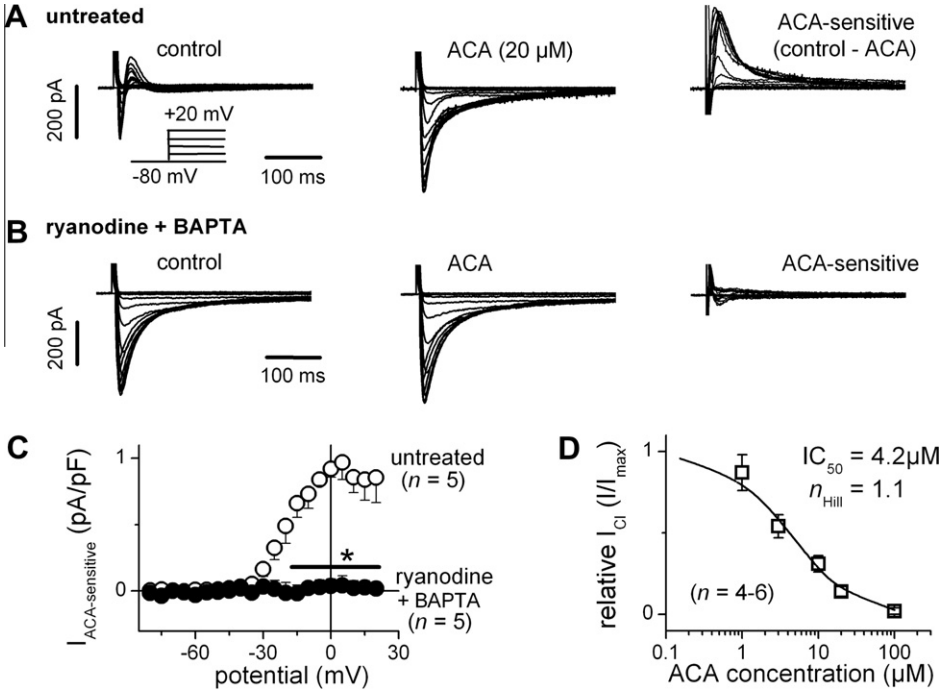

Fig. 2. Suppression of $I_{Cl(Ca)}$ by *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA). (A) Traces of currents induced by depolarizing steps from −80 mV to various levels (between −80 and +20 mV; see inset) in a cell dialyzed with the standard internal solution and superfused with Na^+-free solution. Left panel: the control current. Middle panel: the current during application of ACA (20 μM). Right panel: the ACA-sensitive current calculated as the difference between the control current and that during ACA application. (B) Similar protocol as in (A), but in a cell dialyzed with BAPTA (10 mM)-containing solution and treated with ryanodine (10 μM) in the external solution. (C) Current–voltage relationships of the ACA-sensitive current under control conditions (○) and in BAPTA- and ryanodine-treated cells (●). * Indicates $P < 0.05$ *versus* control in the potential range indicated by horizontal bar (unpaired *t*-test). (D) Effects of different concentrations of ACA on $I_{Cl(Ca)}$ (IC_{50} = 4.2 μM, n_{Hill} = 1.1). Currents are expressed relative to the 100 μM flufenamic acid-sensitive current.

symbols; at potentials $\geqslant -20$ mV, $P < 0.05$ *versus* untreated controls; unpaired *t*-test, $n = 5$). These results indicate that the effect of ACA under standard conditions is due to a suppression of $I_{Cl(Ca)}$, and that the drug has no effect on L-type Ca^{2+} channels. Similarly,

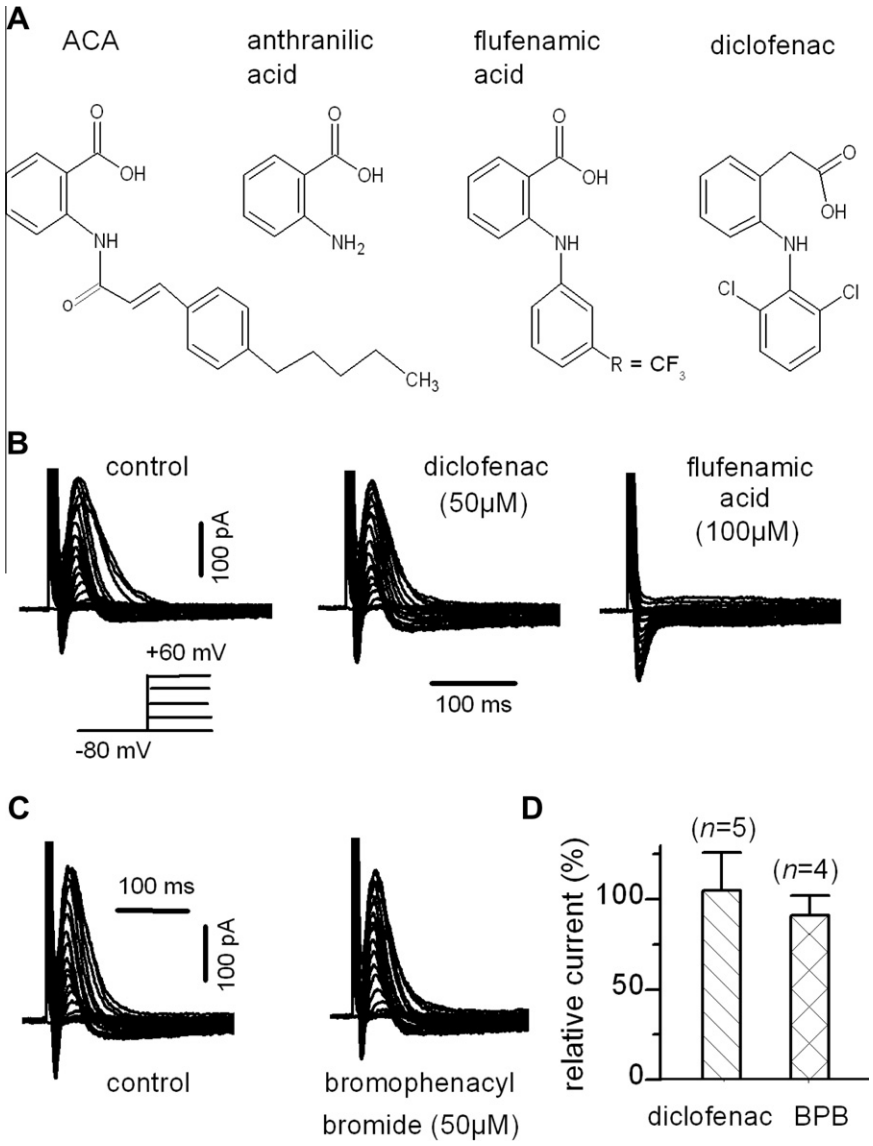

Fig. 3. Effects of diclofenac and bromophenacyl bromide on $I_{Cl(Ca)}$. (A) Chemical structures of ACA (*N*-(*p*-amylcinnamoyl)anthranilic acid), anthranilic acid, flufenamic acid (2-{[3-(trifluoromethyl)phenyl]amino}benzoic acid) and diclofenac (2-[2-(2,6-dichlorophenyl)aminophenyl]acetic acid). Notice the presence of the anthranilic acid moiety in ACA and flufenamic acid, but a modified structure in diclofenac. (B) Traces of currents induced by voltage steps (inset) in a cell dialyzed with the standard internal solution and superfused with Na^+-free solution. Left panel: currents under control conditions. Middle panel: currents during extracellular application of diclofenac (50 μM). Right panel: currents during application of flufenamic acid (100 μM) after diclofenac washout. (C) Similar protocol as in (B), but testing bromophenacyl bromide (BPB, 50 μM). (D) Summary data of drug effects on the transient outward currents. $P = 0.502$ for diclofenac and $P = 0.199$ for BPB, compared with control currents (paired *t*-test).

ACA had no effect on intracellular Ca^{2+} transients induced under voltage clamp (not illustrated).

The concentration-response curve of $I_{Cl(Ca)}$ obtained when using different concentrations of ACA is illustrated in Fig. 2D. The effects of ACA can be described by a 50% inhibitory concentration (IC_{50}) of 4.2 μM and a Hill coefficient (n_{Hill}) of 1.1 ($n = 4$–6 for each concentration).

3.3. Effects of the NSAID diclofenac and the PLA_2 inhibitor bromophenacyl bromide

Given that ACA and flufenamic acid are both fenamates and share an inhibitory action on $I_{Cl(Ca)}$, we wanted to find out whether the fenamate-related drug, diclofenac, which is clinically used as a non-steroidal anti-inflammatory drug (NSAID), also inhibits $I_{Cl(Ca)}$. Diclofenac (50 μM) did not suppress the transient outward current (Fig. 3B; left *versus* middle panels), which at +60 mV was 105 ± 21% of control (Fig. 3D; $P = 0.502$ *versus* control; paired *t*-test, $n = 5$), while in the same cells the current could be suppressed by flufenamic acid (100 μM; Fig. 3B, right panel). Similarly, 100 μM diclofenac was ineffective in two other cells. In addition, because of the known phospholipase A_2 (PLA_2) inhibitory effects of ACA [15], we also tested whether the enzyme could be involved in the action of ACA on $I_{Cl(Ca)}$. The non-selective PLA_2 inhibitor bromophenacyl bromide (50 μM) failed to suppress the transient outward current, which was 91 ± 11% of control (Fig. 3C and D; $P = 0.199$ *versus* control; paired *t*-test, $n = 4$), suggesting that ACA effect on $I_{Cl(Ca)}$ is PLA_2-independent.

3.4. Effects of ACA on the cAMP-activated Cl^- current ($I_{Cl(cAMP)}$)

Having shown that ACA inhibits $I_{Cl(Ca)}$, we wanted to compare its inhibitory effect on $I_{Cl(Ca)}$ with its effect on another Cl^- current, by testing its effect on the cAMP-activated current ($I_{Cl(cAMP)}$), also

present in cardiac ventricular myocytes [20]. For this purpose, nifedipine (100 μM) was included in external solutions to block $I_{Ca,L}$, Na^+-containing, Cs^+-based external solutions and the standard Cs^+-containing pipette solutions were used, and the voltage clamp protocol consisted of ramps. The results are illustrated in Fig. 4. Forskolin, which is known to increase cAMP, induced an outward-rectifying current (−1.4 ± 0.10 pA/pF at −120 mV and 3.9 ± 0.35 pA/pF at +80 mV) with reversal potential of −38.9 ± 1.7 mV (n = 5), practically identical with the calculated equilibrium potential for Cl^- (−40.1 mV) under our experimental conditions. The outward component of the forskolin-activated current could be suppressed by decreasing Cl^-_o, consistent with the view that the current is due to Cl^- influx. ACA (50 μM) decreased the forskolin-activated current to 55 ± 3% at −120 mV and to 68 ± 5% at +80 mV (P < 0.05 compared with the current in forskolin alone; paired t-test, n = 5). The reversal potential for the ACA-sensitive current was −35.6 ± 5.1 mV (P = 0.550 compared with the reversal potential of the forskolin-induced current; paired t-test, n = 5). Fig. 4B shows summary data of the above-mentioned effects and also illustrates the current–voltage relationships. Given that at 50 μM ACA practically fully suppresses $I_{Cl(Ca)}$ (see Fig. 2D), the drug is therefore a less effective inhibitor of $I_{Cl(cAMP)}$ than it is of $I_{Cl(Ca)}$.

4. Discussion

In the present study we show that a Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) is present in pig ventricular myocytes and that it is inhibited by *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA). Our experimental conditions, using Na^+-free external solutions and K^+-free external and internal solutions, were designed to promote high intracellular [Ca^{2+}] levels ([Ca^{2+}]$_i$) and to suppress any eventual K^+-dependent transient outward current, respectively. We show that depolarizing voltage steps to potentials positive to −40 mV induce net inward currents that are short-lived and followed by transient outward currents. The latter currents were eliminated by omitting Cl_o, by externally applying known Cl^- channel inhibitors such as flufenamic acid or DIDS, or by increasing the intracellular buffering of [Ca^{2+}]$_i$ combined with an inhibition of Ca^{2+} release from the sarcoplasmic reticulum. Thus, our data are consistent with previous findings [19] showing the presence of $I_{Cl(Ca)}$ in pig ventricular myocytes.

Transient outward currents (I_{to}) play an important role in the repolarization of the cardiac action potential. In most cases, I_{to} is largely made of K^+ currents through Kv4.x/Kv1.4 channels. However, the expression of these channels differs among species. The currents are large in ventricular cells of rodents, but are absent or very small in ventricular myocytes of pig [17,19] and guinea-pig [21]. There is still controversy concerning the mechanism underlying this lack of Kv4.x/Kv1.4-based currents in pig ventricular cells, with one study reporting lack of protein expression [19], whereas another showed their presence in non-conducting state [22]. Within the heart, the expression of Kv4.x/Kv1.4 channels is also region-dependent, being more prominent in subepicardial layers compared to mid-myocardial or subendocardial layers. The other type of I_{to} found in cardiac cells is due to $I_{Cl(Ca)}$, which we studied here. Because of its nature, $I_{Cl(Ca)}$ is expected to be more prominent under conditions of intracellular Ca^{2+} overload. In a recent study, we showed that ACA depolarizes cardiac myocytes and also prolongs the action potential duration [23]. The latter effect of ACA may be related, at least in part, to the inhibition of $I_{Cl(Ca)}$.

At present, with the molecular identity of Ca^{2+}-activated Cl^- channels and their existence in the human heart still uncertain [24,25], pharmacological agents continue to be a valuable tool in evaluating the contribution of the channels to net ion currents and to membrane potentials [1,10]. Our results show that ACA is an additional useful tool to study $I_{Cl(Ca)}$. The drug reversibly inhibited $I_{Cl(Ca)}$ without affecting $I_{Ca,L}$. This inhibitory action occurred in a concentration range (IC_{50} = 4.2 μM) similar to the inhibition by flufenamic or niflumic acids (IC_{50} range 2–108 μM; see [1]). Despite similar potencies, and despite the effect of ACA on other channels, ACA may be more advantageous than fenamates when studying $I_{Cl(Ca)}$ because some of the latter drugs have been reported to affect $I_{Ca,L}$. The above-mentioned depolarization is due to the induction by ACA of a cation current [23], but this effect also occurs with less potency ($K_{0.5}$ = 24 μM) than the effect on $I_{Cl(Ca)}$.

The mechanism underlying ACA effect on $I_{Cl(Ca)}$ is unclear. ACA is a known PLA_2 inhibitor [15], but its effects on $I_{Cl(Ca)}$ are likely to be PLA_2-independent because they could not be reproduced by the non-selective PLA_2 inhibitor bromophenacyl bromide. ACA has also been recently shown to inhibit some transient receptor potential (TRP) cation channels [26] in a PLA_2-independent manner. Flufenamic acid also inhibits several TRP channels (see [27]), implying a common underlying mechanism of action. Indirect mechanisms of inhibition of $I_{Cl(Ca)}$ involving changes in Ca^{2+} are unlikely given the lack of ACA effect on $I_{Ca,L}$. Rather, because ACA also suppressed cAMP-activated Cl^- currents, the drug could be acting as an anion channel inhibitor. Diclofenac, which is structurally-related to fenamates and to ACA, did not have similar effects on $I_{Cl(Ca)}$, suggesting that structural differences between them may confer specific properties.

In conclusion, the present study shows that ACA suppresses cardiac $I_{Cl(Ca)}$ in a concentration-dependent, but PLA_2-independent

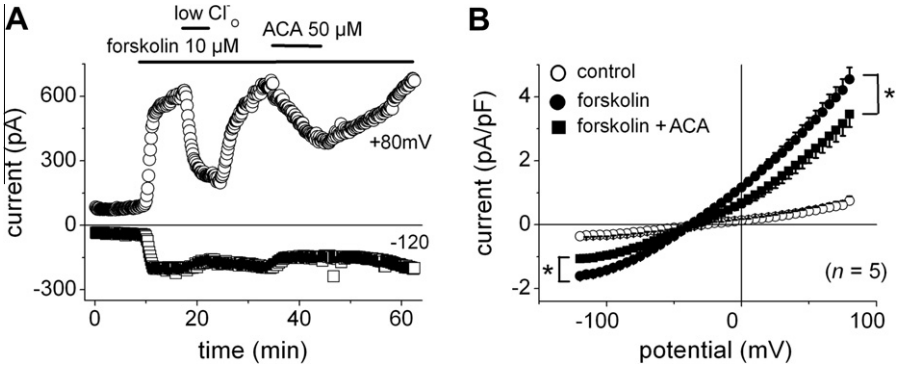

Fig. 4. Effects of ACA on the cAMP-activated chloride current. (A) Currents (at −120 and +80 mV), induced by ramps from −120 to +80 mV in a cell dialyzed with the standard internal solution and superfused with Na^+-containing solution, plotted as a function of time. Forskolin (10 μM) and ACA (50 μM) were applied externally. For low extracellular Cl^-, the anion was replaced by methylsulfate. Periods of drug application and of superfusion with low Cl^- solution are indicated by horizontal bars. (B) Summary data of the current–voltage relationships under control conditions (○), during forskolin application (●), and during ACA application on top of forskolin (■). * Indicates P < 0.05 for currents (at −120 and +80 mV) in forskolin plus ACA *versus* forskolin alone (paired t-test).

manner and without affecting $I_{Ca,L}$. The drug also suppresses cAMP-activated Cl^- currents, but with a lower potency. ACA may, therefore, provide an additional experimental tool or serve as template for the design and development of new drugs useful in modulating anion channels.

Acknowledgments

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