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Pharmacological characterization of muscarinic receptor-activated cation channels in guinea-pig ileum

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1 The pharmacological properties of cationic currents activated by acetylcholine (ACh) (I_{cat}) in guinea-pig ileal smooth muscle cells were investigated, with conventional single patch electrode or nystatin-perforated whole-cell recording. Cs-aspartate was used as the internal solution to allow selective measurement of I_{cat}.

2 Well-known K channel blockers, tetraethylammonium (TEA), 4-aminopyridine (4-AP), procaine and quinine as well as a Ca releasing agent, caffeine, all produced concentration-dependent inhibition of I_{cat} with rapid onset (time constant $\sim$100 ms), when applied externally. The recovery from the inhibition on washout also occurred rapidly in the order of 100 ms except in the case of quinine. Approximate values of the half inhibitory concentrations (IC_{50}) were 10 mM for TEA and caffeine, 1–5 mM for 4-AP and procaine, and 1 μM for quinine. The mode of inhibition was voltage-dependent, i.e., depolarization relieved the inhibition with no change in reversal potential.

3 Externally applied diphenylamine-2-carboxylate (DPC) derivatives, DCDPC and flufenamic acid, produced potent inhibition of I_{cat} at micromolar concentrations (IC_{50}s were <30 μM for DCDPC and 32 μM for flufenamic acid). The onset of and recovery from inhibition occurred slowly and the degree of inhibition depended on the membrane potential only weakly, without any discernible change in the reversal potential.

4 All of the above-tested drugs exhibited comparable inhibitory actions on the voltage-dependent Ca current in the concentration ranges effective at inhibiting I_{cat}. However, amongst them, quinine and flufenamic acid seemed to have several-fold better selectivity for the I_{cat} channel than for the voltage-dependent Ca channel.

5 Internally dialysed GTPγS (100 μM) induced inward cationic currents. The effects of drugs on these currents were similar to their effects on the I_{cat} current.

6 These results clearly indicate that many drugs used as pharmacological tools in smooth muscle research exert considerable nonspecific effects on various types of channels. The mechanism of inhibition and the relevance to use of these drugs as blockers for the I_{cat} channel are discussed.

Keywords: Acetylcholine (ACh); nonselective cation channels; blockers; smooth muscle

Introduction

In mammalian intestinal smooth muscles muscarinic receptor activation leads to at least two major consequences, production of inositol 1,4,5 trisphosphate and membrane depolarization due to increased Na^+ permeability. The latter can be ascribed to the opening of cation (I_{cat}), channels, which serve as the main route for Na^+ as well as Ca^{2+} influx. Although some details of the biophysical nature of the I_{cat} channel have already been published and the involvement of a pertussis toxin-sensitive guanosine 5'-triphosphate (GTP)-binding protein in its activation has been suggested (e.g., Inoue, 1991b), very little effort has been made to characterize the pharmacological properties of this channel, and thus information about selective blockers is lacking.

Recently, it was reported that diphenylamine-2-carboxylate (DPC) derivatives such as 3',5-dichloro-DPC (DCDPC) and flufenamic acid are very potent and relatively selective blockers for Ca^{2+}-activated cation channels in colonic epithelial cells (Gögelein & Capek, 1990). Although the Ca^{2+}-activated cation channels appear to be very different from the I_{cat} channels in mode of activation and voltage-dependence, some essential properties are similar. For example, single channel conductances of both types of channels range between 20–30 pS and the kinetics are slow in both cases (Inoue *et al.*, 1987; Swandulla & Partridge, 1990; Vogalis *et al.*, 1990) as compared with voltage-dependent Na,K and Ca channels. In addition, some of the Ca^{2+}-activated cation channels are reported to be voltage-sensitive and the open probability is increased as the membrane is depolarized (Sturgess *et al.*, 1987; Ehara *et al.*, 1988), as is observed in the case of I_{cat} channel (Inoue & Isenberg, 1990b), and the I_{cat} channel is also sensitive to changes in the intracellular Ca^{2+} concentration (Inoue & Isenberg, 1990c). These observations imply the presence of considerable structural homology between the Ca^{2+}-activated cation channels and the I_{cat} channel. It is therefore of great interest to examine how the above-mentioned blockers for the Ca^{2+}-activated cation channels act on the I_{cat} channel.

In the present study, we have investigated the effects of DPC-derivatives as well as well-known K channel blockers and some other agents which are frequently used as pharmacological tools in electrophysiological studies on excitable membranes. As a result of this work, we found that quinine and DPC derivatives such as DCDPC and flufenamic acid are very potent inhibitors of I_{cat}, and unfavourable nonspecific actions on other types of channels are also evident.

Methods

Cell dispersion and electrical recordings

The procedures used for dispersion of single ileal smooth muscle cells, the system for recording the membrane currents and the method of rapid application of drugs were the same as described previously (Inoue, 1991a; Inoue & Kuriyama, 1993). Briefly, guinea-pigs of either sex weighing 600–1000 g were stunned and exanguinated. Thin sheets of longitudinal

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smooth muscle (1–2 mm × 5–10 mm) were gently stripped off by hand from the terminal part of ileum, then incubated in Ca-free solution containing 0.5–1.0 mg ml^{-1} actinase (Kaken, Tokyo, Japan) and 1 mg ml^{-1} collagenase (type I, Sigma) at 35°C for 15–20 min. The digested sheets of ileal muscle were cut into pieces and triturated in Ca free solution until yielding a sufficient number of cells. Currents were recorded with a commercial patch clamp amplifier (Axopatch 1D, Axon Instruments, Burlingame, CA, U.S.A.) in conjunction with an A/D, D/A converter (Labmaster TL-1 DMA, Axon Instruments), which was run under the control of an IBM compatible personal computer (AMSC-ATC, Alps Electronic, Japan) with 'pClamp v.5.5' programmes (Axon Instruments). All experiments were carried out at room temperature.

Nystatin-perforated whole-cell recording

Patch pipettes (3–5 Mohm) were made from 1.5 mm pyrex glass capillaries. Fresh nystatin stock solution was made every day by dissolving the powder (Sigma) in dimethylsulphoxide (DMSO) to give a concentration of 50 mg ml^{-1}. This was ultrasonicated for about 15 min. The stock solution was further diluted 200–500 times with the internal solution (Cs-aspartate complemented with 1 mM EGTA) and ultrasonicated again for 15 min just before use. Whole-cell recording was achieved with a pipette which was first momentarily dipped in a nystatin-free internal solution and then backfilled with one complemented with nystatin. The timing of the dipping was a critical step to determine the success of forming a 'giga-seal' and subsequent chemical perforation. The other procedures of whole-cell recording were the same as described above.

Solutions

The bathing and pipette solutions were the following (mM): modified Krebs solution: Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, Cl^- 151.4, HEPES/Tris 10 (pH 7.35–7.4); Cs aspartate internal solution: Cs^+ 140, Mg^{2+} 2, Cl^- 24, aspartate$^-$ 120, EGTA10/4~5Ca^{2+} and HEPES 10 (titrated to 7.2–7.25 with Tris base). The Ca-free solution was made by simply omitting Ca^{2+}.

Chemicals

Flufenamic acid and niflumic acid were purchased from Wako (Osaka, Japan). DPC and DCDPC were generous gifts from Dr R. Greger (University of Freiburg). DPC derivates were freshly dissolved into DMSO and then diluted 1000–10000 times by the bathing solution so as to give a final DMSO concentration of no more than 0.1% for any experiment; 0.1% DMSO did not show any effects on its own. The other chemicals used in the present study were purchased

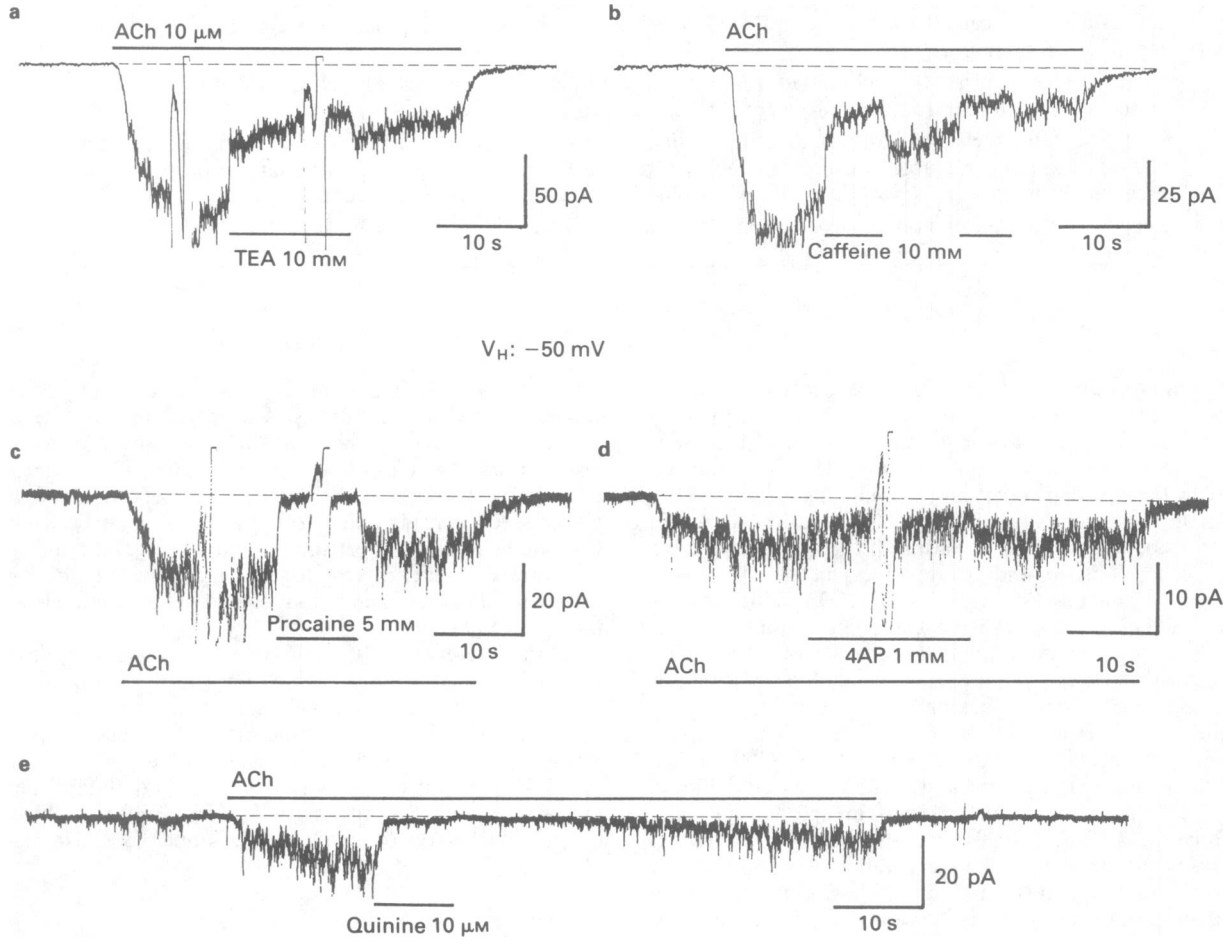

Figure 1 Rapid reduction in the acetylcholine (ACh)-evoked inward currents by K channel blockers and caffeine. Recordings were made with conventional patch electrode whole-cell recording, in which the cell membrane was disrupted by negative pressure after a 'giga-seal' was formed. Throughout the following figures this recording configuration was used, unless otherwise stated (i.e. Figures 4 and 5). The bath and pipette contained modified Krebs solution and Cs-aspartate internal solution, respectively. In the continued presence of 10 μM ACh (indicated by long bars), clamped cells were swiftly moved from the control bathing solution to one containing either tetraethylammonium (TEA) 10 mM (a), caffeine 10 mM (b), procaine 5 mM (c), 4-aminopyridine (4-AP) 1 mM (d) or quinine 10 μM (e) (indicated by short bars). The onset and offset of solution changes are approximate. The speed and efficacy of the rapid solution change are demonstrated in Figure 2. The membrane was held at − 50 mV. Vertical deflections represent slow ramps as shown in Figure 7.

from the following: TEA, indomethacin and nystatin (Sigma), 4-AP (Nacalai, Kyoto, Japan), procaine and caffeine (Wako, Osaka, Japan), quinine and quinidine (Tokyo Kasei, Tokyo). Changes in pH due to tested drugs were minimized by adjusting the pH of stock solutions with addition of Tris base or HEPES as appropriate before use.

Results

The effect of K channel blockers on I_{cat}

As I_{cat} is very sensitive to changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a mixture of EGTA and Ca^{2+} was included in the internal solution to clamp the $[Ca^{2+}]_i$ to 100–200 nM (see Methods) to minimize any secondary changes in the current amplitude and kinetics due to the altered $[Ca^{2+}]_i$ (Inoue & Isenberg, 1990c). This strategy appeared to work satisfactorily since the potentiating effect of voltage-dependent Ca^{2+} influx on the I_{cat} (Inoue & Isenberg, 1990c) was only brief and marginal. Furthermore, membrane depolarizations to − 30 mV–0 mV, which generate a long-lasting Ca inward current and subsequent contraction of smooth muscle cells when $[Ca^{2+}]_i$ is buffered weakly, did not contract cells examined over 1 min. Under these conditions, however, the inward currents evoked by 10 μM ACh showed a decay to a varying extent, with a time constant of 30–60 s, and underwent a strong rundown on repeated application. Therefore, we first assessed the effects of blockers on I_{cat} with a fast solution exchange device based not on sequential but on a single administration of ACh as performed previously (Inoue, 1991a).

Figure 1 demonstrates the time course of several well-established blockers on I_{cat}. K channel blockers such as TEA, 4-AP and procaine all caused clear inhibition. The inhibitions seemed to occur rapidly within about 50–100 ms, the speed of which compares to that of solution exchange as demonstrated by Na removal in Figure 2. The inhibitory action of these agents was reversible because the degree of inhibition evaluated at onset and recovery from blockade was in close agreement. For example, with TEA 10 mM, the percentage inhibition was 37% and 35% respectively at onset and recovery (Figure 1a). Caffeine, a Ca^{2+} releaser from the sarcoplasmic reticulum (SR), also produced inhibition of I_{cat} at a concentration of 10 mM, the nature of which appeared similar to that produced by the K channel blockers (also see below). Figure 3a summarizes the results pooled from many cells with various concentrations of these blockers. The half inhibitory concentrations (IC_{50}s) were between 2 and 5 mM for 4-AP, 1 and 5 mM for procaine, and about 10 mM for TEA and caffeine. It should be noted that all the IC_{50} values

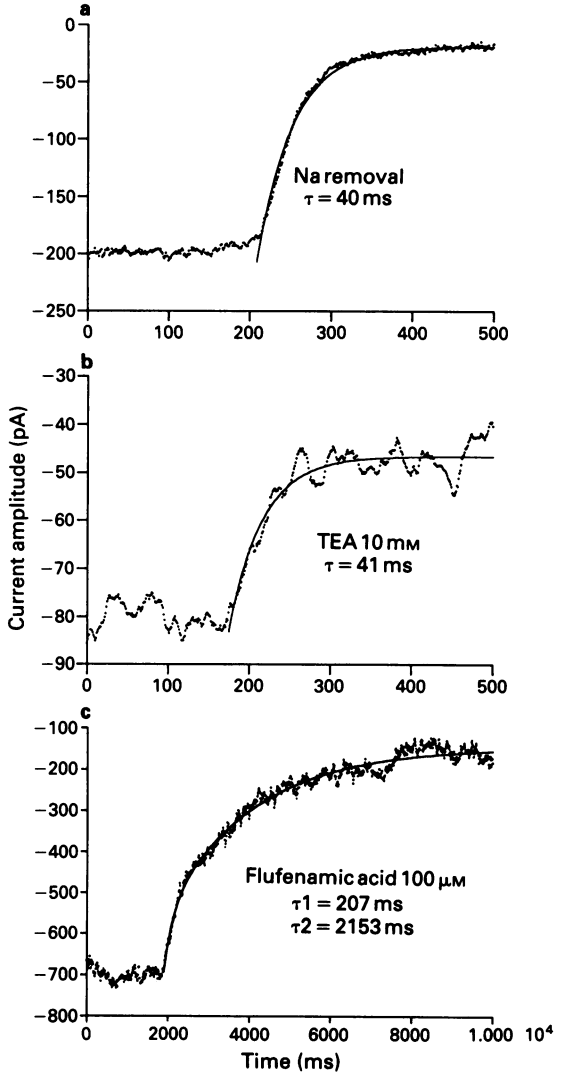

Figure 2 Efficiency of the rapid solution changing system evaluated by Na removal and the onset of tetraethylammonium (TEA) or flufenamic acid induced inhibition of the acetylcholine (ACh)-evoked inward current. (a) At a holding potential of − 50 mV, Na^+ was removed from the bathing solution, by switching from normal Krebs solution to Na^+-free Krebs solution (Tris replacement) in the continued presence of 10 μM ACh. The time constant of decrease in the I_{cat} amplitude is 40 ms in this experiment (on average, 39 ± 9 ms, $n = 4$). On reintroduction of Na^+, the current recovered with the time constant of 42 ± 5 ms ($n = 4$). The slower time constant of the solution change as compared to a similar method applied to rat and bullfrog sensory neurons (ca. 10 ms; Bean, 1990) may be due to the spindle shape of ileal smooth muscle cells. (b and c) The time course of inhibition of the ACh-evoked current by 10 mM TEA (b) or 100 μM flufenamic acid (c). Note that the time constant for TEA-induced inhibition (41 ms) compares to that for Na removal, while that for flufenamic acid is considerably slower (fast and slow time constants are 207 and 2153 ms, respectively). The fast time constant for flufenamic acid was not always observed.

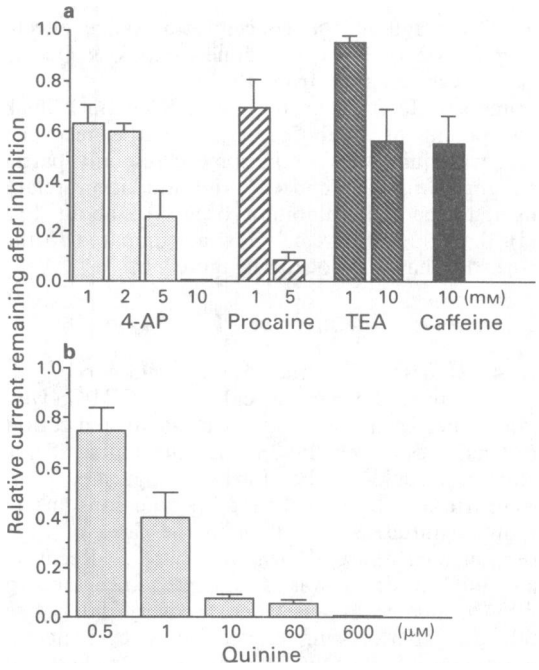

Figure 3 Concentration-dependence of the inhibition of the acetylcholine (ACh)-evoked inward current by K channel blockers and caffeine. The results obtained from experiments similar to those shown in Figure 1 are summarized. Each column represents mean (and s.e.) calculated from 5–10 experiments by measuring the fraction of the ACh (10 μM)-evoked current which remained in the presence of the tested drug. In the case of quinine (b), the best fit using the equation in Figure 5 gave values of 0.84 μM and 1.94 for K and n, respectively.

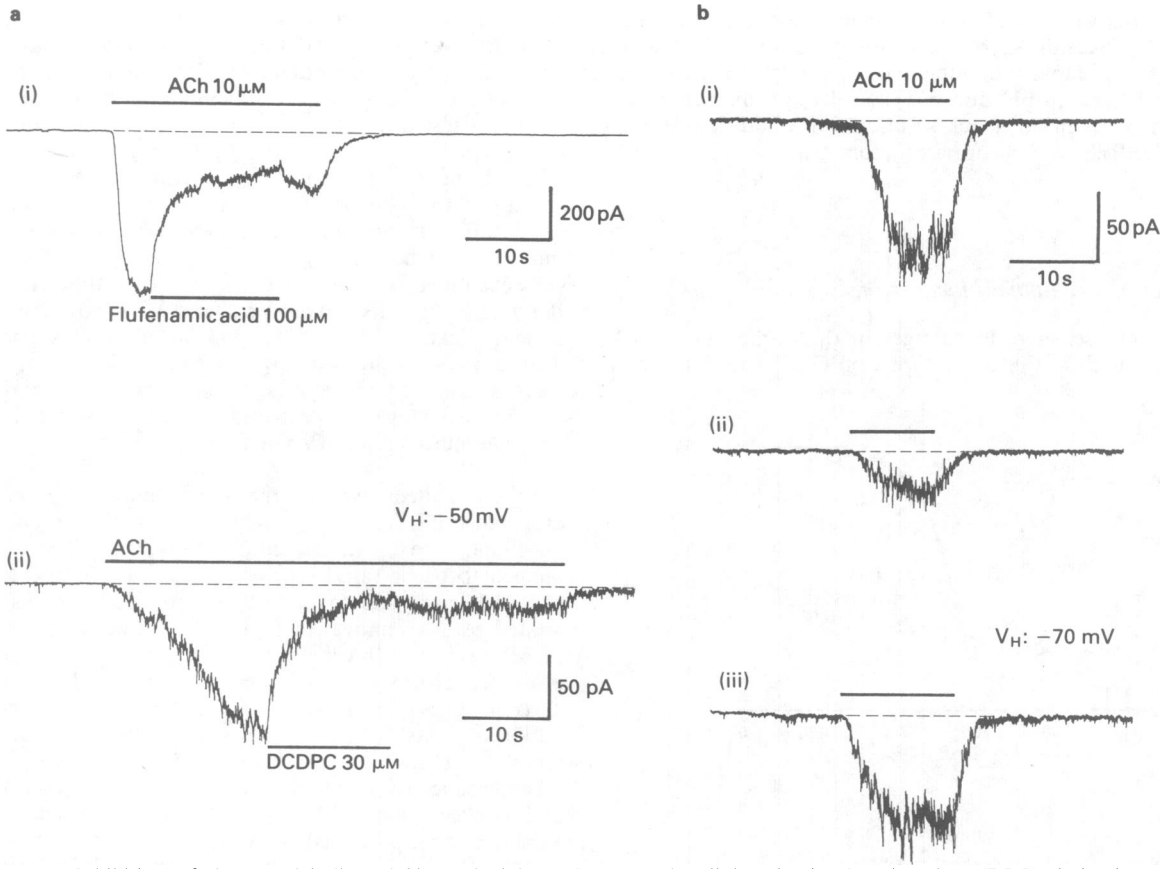

Figure 4 Inhibition of the acetylcholine (ACh)-evoked inward current by diphenylamine-2-carboxylate (DPC) derivatives. (a) Currents were recorded with conventional whole-cell recording. ACh and DPC derivatives were applied at bars with a fast solution change. Conditions used here are the same as in Figure 1. (b) Currents recorded with the nystatin-perforated whole-cell recording before (b(i)), after 10 min pretreatment with (b(ii)) and 10 min after washout of (b(iii)) 50 μM flufenamic acid. Flufenamic acid was added in the bath with ordinary bath superfusion, while 10 μM ACh was rapidly applied at bars with a fast solution exchange device described in Figure 1 in the continued presence of flufenamic acid. The holding potential was − 70 mV. ACh 10 μM was added during the bars.

obtained here fall in the concentration-range effective for blocking certain types of K channels (Cook & Quast, 1990) or causing Ca^{2+} release from the SR.

Quinine is different from the above K channel blockers. It is very potent at inhibiting I_{cat}, but the recovery after washout was quite slow or in some cases only partial over 30 s (Figure 1e). The concentration-inhibition relationship reveals that the half inhibition occurs at about 1 μM (see legend); the value is extremely low as compared to those for the other K channel blockers (Figure 3b).

DPC derivatives potently block I_{cat}

Figure 4a shows actual traces of inhibition of I_{cat} by two DPC derivatives, flufenamic acid and DCDPC, which are reported to be potent blockers of Ca^{2+}-activated cation channels (Gögelein & Capek, 1990). The time course of inhibition was slow and could not be clearly distinguished from those of desensitization. It seemed that the time constant was one order of magnitude slower than in the cases of K channel blockers such as those shown in Figure 2. Recovery after washing out the drugs was incomplete, since the degree of inhibition evaluated at onset and recovery did not seem to coincide. For this reason, we decided to evaluate the inhibitory potency of the DPC derivatives on repeated application of ACh employing the nystatin-perforated whole-cell recording method (Horn & Marty, 1988; also see Methods).

Figure 4b shows actual current traces before, during and after the application of 50 μM flufenamic acid with nystatin-perforated whole-cell recording. At a holding potential as negative as − 70 mV with Cs^+ in the pipete solution, contamination of I_{cat} with another possible inward current, e.g. a Ca^{2+}-activated Cl^- current, seemed negligible because the reversal potential of the inward current evoked by 10 μM ACh did not shift significantly on halving the extracellular Cl^- concentration (8.0 ± 5.4 mV for normal Krebs ($n = 11$) vs. 8.6 ± 2.9 mV for 75 mM Cl Krebs ($n = 4$)). Under these conditions, time-dependent reduction in the I_{cat} amplitude was no more than 20% over 30 min on repeated applications of 10 μM ACh with an interval of 5–10 min (also see Figure 1 in Inoue & Kuriyama, 1992), otherwise the data were discarded. This greatly reduced errors of overestimating the effects of blockers, which may arise from rundown. As shown in Figure 4b (ii), administration of 50 μM flufenamic acid for 10 min resulted in reduction in the I_{cat} current by 70%. This inhibitory effect persisted over a few minutes after the washout of flufenamic acid, and was evident when the drug was administered for several minutes and then washed out just before the application of ACh. The latter observation excludes the involvement of open channel blockade mechanism reported for e.g., MK801 which is known as a potent and selective blocker of NMDA-type cation channels (Huetter & Bean, 1988). Figure 5 summarizes the concentration-dependent inhibition caused by various DPC derivatives. The IC_{50}s are smaller than 30 μM for DCDPC but larger than 100 μM for DPC and niflumic acid (Figure 5a). The concentration-inhibition curve for flufenamic acid gives an IC_{50} of 32 μM and a cooperativity number of one (open circles in Figure 5b).

Effect of blockers of the I_{cat} *channel on the voltage-dependent Ca channel*

The selectivity of these agents was investigated in a study of their effects on the voltage-dependent Ca channels. Figure 6a illustrates the current-voltage relationship in the presence and

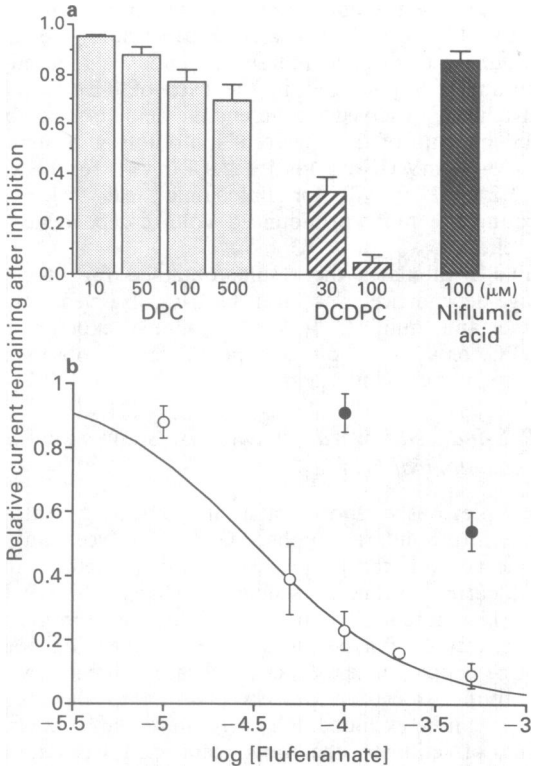

Figure 5 Concentration-dependence of the inhibition of the acetylcholine (ACh)-evoked inward currents by diphenylamine-2-carboxylate (DPC) derivatives. The results of nystatin-perforated whole-cell recording seen in Figure 4b are summarized. The degree of inhibition was calculated as the ratio of I_{cat} current amplitude in the presence of a drug tested versus that in control response. Control responses were obtained before and after application of DPC derivatives, in order to check the rundown of currents (see text), and the first response was used as the reference. (a) Each column represents mean (+s.d.) calculated from 3–6 cells, respectively. (b) Concentration-inhibition curve of the ACh (10 μM)-evoked inward current for flufenamic acid (○). The curve was fitted by an empirical Hill type equation $(1/(1+([\text{flufenamic acid}]/K)^n)$ where [flufenamic acid], K and n denote the concentration of flufenamic acid, the concentration of flufenamic acid to produce 50% inhibition and the cooperatively number. The best fit of the data points (○) gave K = 32 μM and n = 1; (●) represent the fraction of voltage-dependent Ca currents remaining in the presence of flufenamic acid. The currents were measured either with voltage-ramps (see Figure 6a) or by depolarizing pulses. Data points were obtained from 3–7 cells.

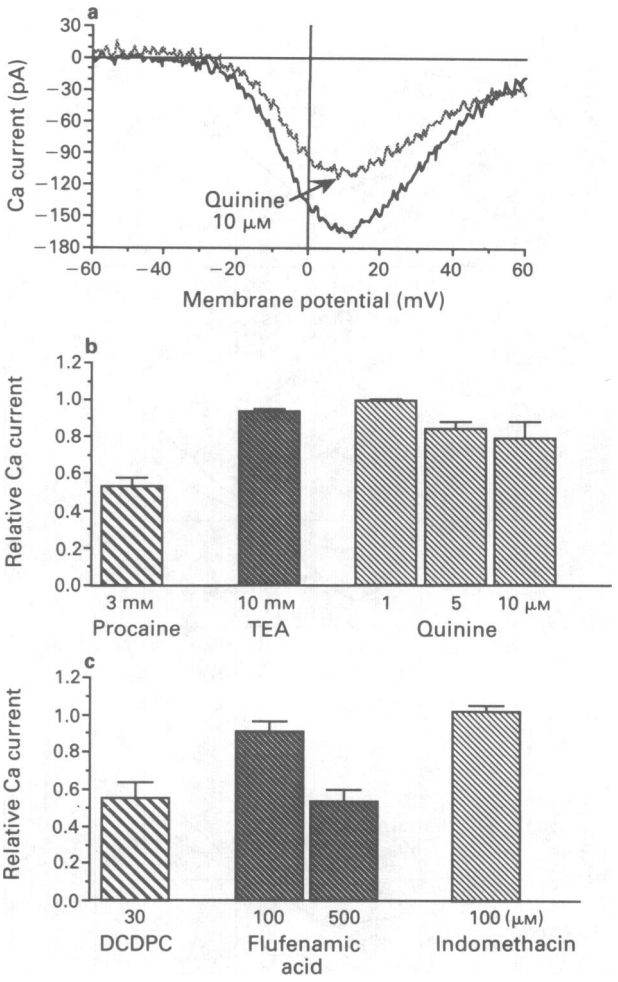

Figure 6 Inhibition of voltage-dependent Ca channels by various drugs. The bath contained modified Krebs solution and the pipette Cs-aspartate solution in which only 10 mM EGTA, instead of EGTA/Ca mixture, and 2 mM ATP were added. (a) An 800 ms voltage ramp (− 120 to 80 mV) was applied at a rate of 0.25 V^{-1} to activate voltage-dependent Ca currents. Leak currents due to depolarization were obtained in the presence of 5 μM nicardipine and subtracted. The currents were plotted against membrane potential as shown in Figure 7. (b and c) The fraction of Ca current remaining in the presence of the drugs named in the figure.

absence of 10 μM quinine, evaluated by a 800 ms ramp pulse. The presence of quinine resulted in reduction of the voltage-dependent Ca current, the extent of which was virtually voltage-independent. As summarized in Figure 6b, quinine reduced the voltage-dependent Ca current by no more than 20% at a concentration of 10 μM, which inhibited I_{cat} by more than 90% (Figure 3b). In contrast, 3 mM procaine produced inhibition of the voltage-dependent Ca current and I_{cat} to a similar extent.

With DPC derivatives, 30 μM DCDPC, which is the most potent at inhibiting I_{cat}, also caused a comparable inhibition of the voltage-dependent Ca current, whereas 100 μM flufenamic acid, which produces ca. 80% reduction in I_{cat}, affected the voltage-dependent Ca current only slightly (Figure 6c and filled circles in Figure 5b).

These results indicate that quinine and flufenamic acid are the preferred blockers among the above-tested drugs against I_{cat} channel with regard to selectivity on the voltage-dependent Ca channels. The ratio of the inhibitory effect on I_{cat} versus that on the voltage-dependent Ca current is 4.5 and 7.9 for 10 μM quinine and 100 μM flufenamic acid respectively, while this value is near to 1.0 in the case of 30 μM DCDPC (1.47).

Influence of blockers on the ionic selectivity and voltage-dependence of the I_{cat} current

The mechanisms underlying the inhibitory actions of the above-mentioned agents on I_{cat} may be complicated, e.g., the onset and recovery time course seem to be different among different agents as shown in Figure 2. In order to get further insight, we compared the voltage-dependence and ionic selectivity of the I_{cat} current before and after the applications of blockers with ramp command voltages. This approach is practical because only one ramp instead of a set of step pulses allows not only measurement of the reversal potential but a more complete delineation of the voltage-dependence of the I_{cat} current. As illustrated in Figure 7a (the uppermost trace), the membrane was first held at − 120 mV for 250 ms, to set the I_{cat} current in a steady state (time constant of relaxation at this potential is 40–50 ms, Inoue & Isenberg, 1990b), and then depolarized by a ramp at a rate of 0.1 $V\,s^{-1}$. The net current corresponding to this voltage change in the absence of ACh was nearly ohmic (the middle pale trace), but changed to an inverted bell-shaped curve in the presence of ACh (the bottom thick trace). The current component sensitive to ACh was then defined as a difference of net currents in the presence and absence of ACh (i.e., the

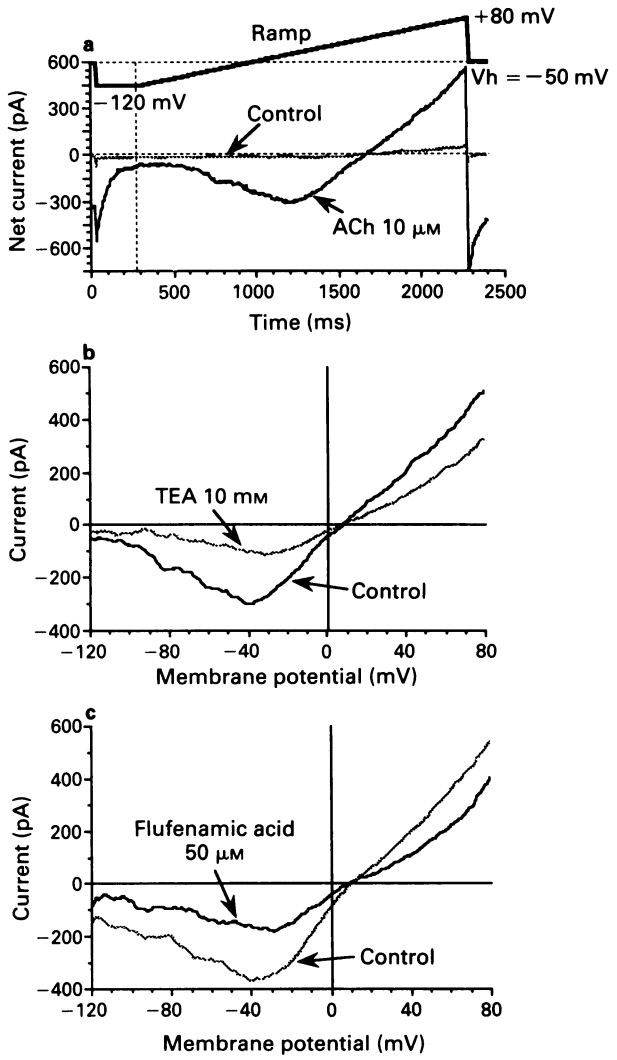

Figure 7 Changes in the current-voltage relationships of the acetylcholine (ACh)-evoked inward current by addition of tetraethylammonium (TEA) or flufenamic acid. The bath and pipette conditions were the same as in Figure 1. (a) The voltage ramp protocol employed. Three traces represent a voltage change (uppermost bold line), corresponding currents in the absence (middle pale) and the presence (bottom thick) of 10 μM ACh, respectively. In order to block the voltage-dependent Ca current, 5 μM nicardipine was present. (b and c) The difference currents (bottom trace − middle trace in a) were converted into current-voltage curves.

bottom trace − the middle trace). Figure 7b and c plot the ACh-sensitive currents obtained in this way against membrane potential. The validity of this method of assessing the voltage dependency was verified by the close agreement of Boltzmann parameters obtained by the two different voltage protocols under the same ionic conditions: the half maximum activation potential (V_h) and the slope factor (k) were -47.9 ± 4.9 mV and -18.0 ± 3.7 mV for the above ramp protocol ($n = 10$) and -45.9 ± 4.6 mV and -21.4 ± 2.1 mV for the multiple step pulse protocol, respectively (see Figure 8 of this paper and Table 1 in Inoue, 1991a).

Both TEA 10 mM and flufenamic acid 50 μM flattened the current-voltage relationship for I_{cat} but the reversal potential was unaffected (Figure 7b and c). As summarized in Table 1, no significant shift of reversal potential was observed in the presence of any blocker listed. This suggests that the blockers do not change the ionic selectivity of the I_{cat} channel.

The steady state activation curves calculated from the current-voltage relationships were shifted to the right in the presence of TEA, while little shift was observed in the presence of flufenamic acid: the shift of V_h value (ΔV_h) was only a few mV for flufenamic acid but amounted to 12 mV for TEA (Figure 8a(ii) and a(i)). Furthermore, the degree of inhibition at a given membrane potential is reduced as the membrane is depolarized in the case of TEA, while only small relief is observed for flufenamic acid (Figure 8b(i) and b(ii)). The ratio of the degree of inhibition at + 80 mV over that at − 80 mV (I_{80}/I_{-80}) is 1.9 ± 0.4 ($n = 4$) for TEA 10 mM and 1.2 ± 0.2 ($n = 3$) for flufenamic acid 50 μM, clearly indicating the more pronounced voltage-dependence of the TEA effect.

Table 1 tabulates the results obtained for other drugs. Voltage-dependence was also clearly observed for 4-AP, caffeine and quinine. In one available experiment with DCDPC, only weak voltage-dependence was observed as in the case of flufenamic acid.

GTPγS-induced inward currents also show a similar pharmacological profile

It was previously reported that in guinea-pig ileal smooth muscle intracellularly applied GTPγS induces an inward cationic current, the properties of which resemble those of the I_{cat} current (Inoue & Isenberg, 1990a; Komori & Bolton, 1990). This curent is thought to be induced through persistent activation of a pertussis toxin-sensitive G-protein that might be coupled to the I_{cat} channel rather directly, where the association-dissociation process of ACh to the muscarinic receptor can be excluded. We therefore tested whether or not the sties of actions of the above-mentioned drugs are located downstream of the receptor, by observing their effects on the GTPγS-induced inward current. After the start of internal dialysis with 100 μM GTPγS, noisy inward currents were observed and reached a level ranging from − 10 to − 75 pA (-35 ± 21 pA, $n = 10$) within 10–20 min. Usually, these currents were maintained for 30–60 min and declined slowly. As shown in Figure 9, addition of 5 mM procaine, 10 μM quinine or 30 μM DCDPC in the bath suppressed the noisy inward current almost completely, while 10 mM TEA and 10 mM caffeine (data not shown) reduced the current to about the half of the original amplitude. After washout of the drugs, recovery from the inhibition occurred in several minutes with quinine and DCDPC. Quinidine (10 μM), which is a stereoisomer of and equipotent with quinine, also markedly diminished the GTPγS-induced inward current. Other classes of K channel blockers such as charybdotoxin and glibenclamide showed no discernible effects on the GTPγS-induced currents.

The inhibitory potencies and time courses of the drugs tested are consistent with those described for the I_{cat} current. This supports the hypothesis that the drugs act somewhere downstream of the receptor, most likely at the level of the I_{cat} channel protein.

Discussion

The main findings of the present study are: (1) many agents used as pharmacological tools in smooth muscle research including TEA, 4-AP, procaine, quinine and caffeine are all able to inhibit the I_{cat} channel at concentrations in normal use, but micromolar quinine might be useful to block selectively the I_{cat} channel. (2) Potent blockers for the Ca^{2+}-activated cation channel, e.g. DPC derivatives, are also effective at inhibiting the I_{cat} channel at micromolar concentrations. The observed effects are likely to be actions on the I_{cat} current itself, since any indirect effects of these agents were minimized with internal dialysis with Cs aspartate and clamping $[Ca^{2+}]_i$ with a Ca^{2+}/EGTA mixture. The mode of inhibition of the various agents appears to differ in time course and voltage-dependence and will be discussed below. Such differences apparently divide the agents into two groups, fast (K channel blockers and caffeine) and slow blockers (DPC derivatives).

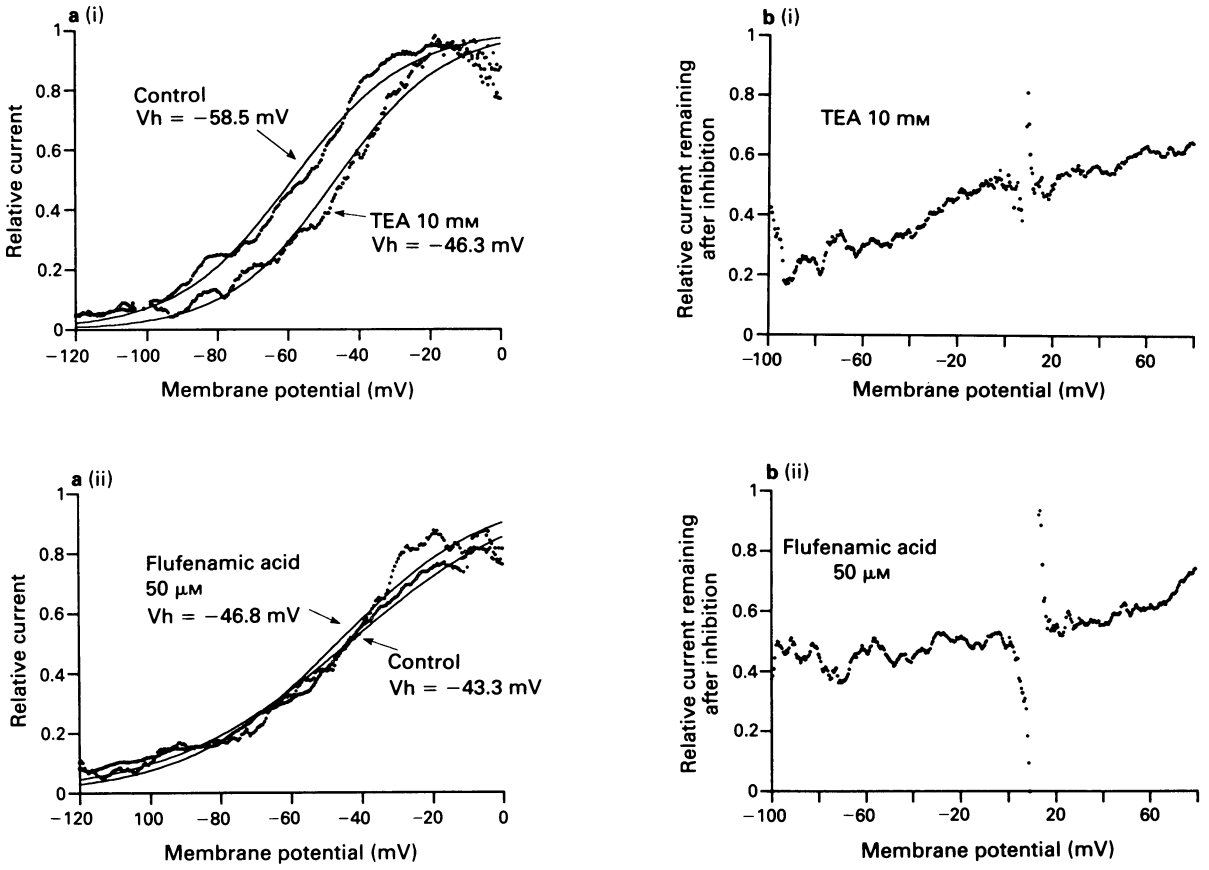

Figure 8 Steady state activation curve and voltage-dependence of the acetylcholine (ACh)-evoked inward current in the presence of either tetraethylammonium (TEA) or flufenamic acid. (a) Examples of the steady state activation curve of I_{cat} evaluated from Figure 7b and c. The chord conductance, the ratio of the I_{cat} amplitude over the driving force (the membrane potential − the reversal potential), was calculated at a given membrane potential and then normalized to its maximum. At membrane potentials positive to 0 mV, which are near to the reversal potential, serious scatter in calculated values were observed due to the small current amplitudes and driving forces, thus such data were not included in the evaluation. Smooth sigmoid curves represent theoretical ones drawn from using parameters of the best fit according to the Boltzmann equation $1/(1 + \exp((Vm-Vh)/k))$, where Vm, Vh and k denote the membrane potential, the half-maximum activation potential and the slope factor, respectively. (b) Changes in the degree of inhibition by TEA 10 mM (i) or flufenamic acid 50 μM (ii) as a function of the membrane potential.

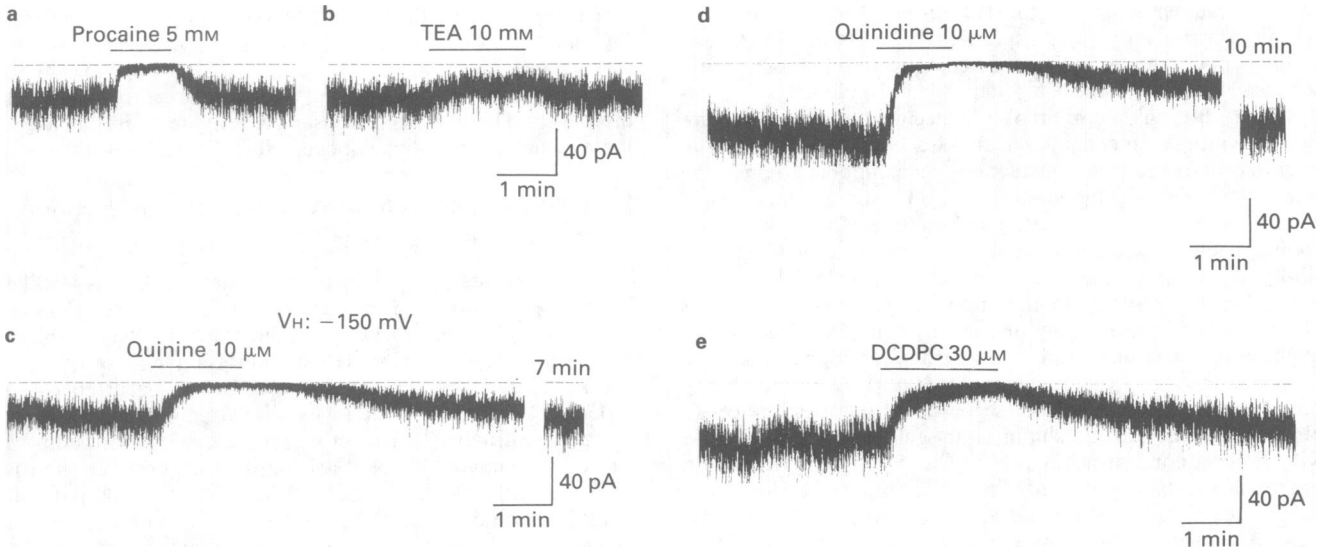

Figure 9 Effects of various drugs on the GTPγS-induced inward current. The same conditions as in Figure 1 were used except that 100 μM GTPγS was included in the pipette. At a holding potential of − 50 mV, various drugs were superfused into the bath at a rate of 0.07–0.08 ml s^{-1} (the chamber volume is 0.2 ml).

Table 1 Comparison of the reversal potential, voltage-dependence of the inhibitory effect (I_{80}/I_{-80}) and shift in the half-maximum activation potential (Δ Vh) in the presence of various drugs

	Erev (mV)	I_{80}/I_{-80}	Δ *Vh* (mV)
Control	8.0 ± 5.4 (11)	NA	NA
TEA (10 mM)	6.8 ± 1.6 (4)	1.9 ± 0.4 (4)	14.6 ± 2.7 (3)
Caffeine (10 mM)	5.2 ± 1.7 (3)	1.9 ± 0.5 (3)	20.7
4-AP (1 mM)	10.9 ± 1.6 (4)	3.1 ± 0.7 (4)	13.9 ± 2.2 (3)
Quinine (1 μM)	9.8 ± 1.4 (2)	2.2 ± 0.3 (3)	10.7 ± 2.9 (2)
Flufenamic acid (50 μM)	6.1 ± 5.6 (3)	1.2 ± 0.2 (3)	4.0 ± 2.3 (3)
DCDPC (30 μM)	5.7	1.1	0.5

The numbers in parentheses represent the number of experiments. For the definition of I_{80}/I_{-80} see the text. Control indicates the modified Krebs solution without drugs. NA indicates no available data. For abbreviations, see text.

K channel blockers and caffeine as nonspecific blockers

As generally documented in the literature, TEA in millimolar concentration is able to block several distinct classes of K channels including Ca^{2+}-dependent K channels, the delayed rectifier K channel, and the ATP-sensitive K channel (Cook & Quast, 1990). Amongst them, a large conductance K channel (designated as the maxi-K channel) is the most susceptible to TEA applied both externally and internally, the IC_{50} for the external TEA being as low as 0.3 mM in various smooth muscles. Millimolar concentrations of 4-AP are also reported to block the delayed rectifier (e.g. Beech & Bolton, 1990) as well as fast inactivating voltage-dependent K currents (A-like currents; Okabe *et al.*, 1987; Beech & Bolton, 1989; Imaizumi *et al.*, 1990; Smirnov & Aaronson, 1992). Procaine (1–10 mM), together with other local anaesthetics, blocks not only a broad range of voltage-dependent channels (Na and K channels, Cook & Quast, 1990; Ca channels, the present work) but also the Ca^{2+} release process from the internal stores of smooth muscles (Itoh *et al.*, 1981). Caffeine (5–20 μM) is frequently used as an experiment tool to mobilize Ca^{2+} into the cytosol from the SR (Iino, 1989), but also inhibited the I_{cat} channel (IC_{50} is c.a. 10 mM) not due to its Ca^{2+} releasing action since the $[Ca^{2+}]_i$ was clamped in the range of 100–200 nM in the present experiments. These observations clearly indicate that the effective concentration ranges of K channel blockers and caffeine considerably overlap those which inhibit the I_{cat} channel, and thus emphasize the need of special care for designing and interpreting experiments with the aid of such pharmacological agents.

Quinine seems to be one of the most desirable blockers of the I_{cat} channel from the present results, but the literature reveals a variety of its nonspecific actions (Cook & Quast, 1990). For example, it is reported that quinine is a fairly selective blocker of the Ca^{2+}-dependent K channels (IC_{50} is > 10 μM) but in submillimolar concentrations it also suppresses voltage-dependent Na and Ca channels. Further, in angiotensin II-induced contractions, a component remaining after blockade of voltage-dependent Ca or K channels is found to be further inhibited by quinine (Cook & Quast, 1990). These observations seem to exclude largely the possibilty of using quinine as a selective I_{cat} channel blocker. However, it deserves attention that the reported IC_{50} values against other currents are not lower than 5 μM, most frequently 10 μM–1 mM, and are several fold higher than the IC_{50} obtained for the I_{cat} current (1 μM). This raises the possibility that quinine can serve as a relatively selective blocker for the cation channels in guinea-pig ileal muscle with limited concentration ranges (ca. 5–10 μM). In fact, in preliminary experiments with the intracellular recording technique, 10 μM quinine abolished depolarizations evoked by 1 μM ACh with only modest changes in spike discharge.

The effects of DPC derivatives on the I_{cat} channel

The inhibitory potencies of DPC derivatives on the I_{cat} channel accord approximately with those observed against Ca^{2+}-activated cation channels by use of single channel recordings (Gögelein & Pfannmüller, 1989; Gögelein *et al.*, 1990; Siemer & Gögelein, 1992). The sequence of inhibitory efficacy is DCDPC > flufenamic acid >> DPC ⩾ niflumic acid and the IC_{50} for DCDPC or flufenamic acid is about 10 μM, although the tested drugs were administered from the outer side of the cell membrane whilst most of data for the Ca^{2+}-activated cation channels were obtained in the inside-out patch configuration. In rat exocrine pancreatic cells, DPC derivatives block Cl conductances with comparable efficacy to Ca^{2+}-activated cation channels (Gögelein & Pfannmüller, 1989). However, conflicting evidence has been provided on the inability of 10 μM DCDPC to affect Cl channels in distal colonic epithelial cells of the same species (Gögelein & Capek, 1990). In our experiments, we did not observe the effects of these drugs on the Cl conductance in the guinea-pig ileum, since such a conductance could hardly be detected under the experimental conditions employed. However, the voltage-dependent Ca current showed almost the same sensitivity to 30 μM DCDPC as did the I_{cat} current. Furthermore, 100 μM flufenamic, DPC and DCDPC exerted inhibitory effects on the transient outward currents during depolarizing pulses, the properties of which are similar to those described e.g. by Benham & Bolton (1986) and Ohya *et al.* (1987) (Chen, Inoue & Ito, unpublished data). These observations suggest that internal Ca stores are also a major target of these drugs, since Poronik *et al.* (1992) have recently provided evidence that several synthesized DPC derivatives including flufenamic acid and DPC possess the capability of provoking calcium transients in the absence of external calcium. In addition, we noticed in our preliminary microelectrode experiments that 30 μM–100 μM flufenamic acid and DCDPC abolish slow membrane oscillations (the so-called slow waves, Bolton, 1979), resulting in membrane hyperpolarizations of 10–20 mV and subsequent cessation of spike discharge. These observations obviously restrict the use of these drugs as selective blockers for the I_{cat} current.

Possible mechanisms involved in the action of blockers for the I_{cat} current

From the results of some mechanistic approaches undertaken in the present study, at least two important different features have become evident between caffeine and K channel blockers, and DPC derivatives. First, inhibition and recovery of the I_{cat} current by the former drug group occurred in the order of 100 ms, while those by the latter were considerably slower (see Figure 2). Secondly, the degree of inhibition was strongly attenuated by depolarizing the membrane in the case of K channel blockers and caffeine but was relieved only weakly in the cases of DPC derivatives. In addition, the site of action of blockers is not on the muscarinic receptor but probably on the I_{cat} channel itself from the observations on GTPγS-induced inward currents. One possible explanation may be as follows. In cation-permeable channels, large positively charged molecules such as TEA, in the extracellular space, are believed to be driven into the orifice or

attracted near to a negatively charged binding site located midway down the channel pore along the transmembrane potential gradient, and to interfere with the permeation of cations (e.g. Hille, 1984). Such interference could occur rapidly due to their easy access and be relieved by depolarization of the membrane due to reduction of the potential gradient which might slow the binding process of the drug molecules. On the other hand, DPC derivatives possess high lipophilicity and are highly membrane-permeable, although most of the molecules are negatively charged at a physiological pH (Gögelein & Phannmüller, 1989). This might enable the drugs to pass through the plasma membrane to an internal site. This mechanism seems consistent with slow onset and recovery of their inhibitions. Indeed, the inhibitory effect of flufenamic acid or DCDPC on the I_{cat} currents persisted for several minutes even after washout of the drugs, and their efficacies on the I_{cat} channel are similar to those on the Ca^{2+}-activated cation channels, where the drugs are applied at the intracellular side of the plasma membrane. In addition, this view is consistent with the finding that DPC derivatives decrease the open probability of the Ca^{2+}-activated cation channels but such an effect is not associated with the appearance of flickering within long opening intervals, which is usually observed for open channel blockade (Gögelein & Capek, 1990).

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