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Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig)

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Abstract. 1. Cells were isolated by incubating chunks of tissue from the urinary bladder of the guinea-pig in a high potassium, low chloride medium containing 0.2 mM calcium plus the enzymes collagenase and pronase. After isolation, the cells were superfused with a physiological salt solution (PSS) containing 150 mM NaCl, 3.6 mM $CaCl_2$ and 5.4 mM KCl (35° C). Patch electrodes filled with an isotonic KCl-solution were used for whole cell recordings. With a single electrode voltage clamp we measured a capacitance of 50 ± 5 pF per cell, an input resistance of 200 ± 25 kOhm $\cdot$ cm^2 and a series resistance of 44 ± 4 Ohm $\cdot$ cm^2.

2. The cells had resting potentials of -52 ± 2 mV. They did not beat spontaneously but responded to stimuli with single action potentials (APs) which rose from the threshold (-38 mV) with a maximal rate of 6.5 ± 1.8 V/s to an overshoot of 22 ± 3 mV. The AP lasted for 36 ± 4 ms (measured between threshold and -40 mV). Continuous cathodal current produced repetitive activity, a pacemaker depolarization followed the AP and preceded the next upstroke.

3. Net membrane currents evoked by clamp steps to positive potentials were composed of an inward and an outward component. The inward component generating the upstroke of the AP was carried by Ca ions (i_{Ca}, Klöckner and Isenberg 1985). The repolarization resulted from a potassium outward current i_K. Ca-channel blockers (5 mM $NiCl_2$) reduced i_K suggesting that (part of) i_K was Ca-activated.

4. i_K rose within about 100 ms to a peak of $40-200$ μA/cm^2 from which it inactivated slowly and incompletely. The inactivating i_K followed a bell-shaped voltage-dependence, the noninactivating i_K an outwardly rectifying one. Both parts had similar steady state inactivation curves with a half maximal inactivation potential at -36 mV and a slope of 9 mV.

5. Repolarization to -50 mV induced outward tail currents which reversed polarity at -85 mV (the calculated potassium equilibrium potential). The amplitude and the time course of the envelope of the tail currents varied in proportion to i_K during the prestep. Thus, the tail current is suggested to reflect the turning off of a potassium conductance which had been activated during the prepulse.

6. i_K was largely reduced but not blocked by 20 or 150 mM tetraethylammonium (TEA). TEA did not significantly change the resting potential, but it prolonged the AP and facilitated upstroke and overshoot. i_K could be blocked by loading the cells with Cs released from Cs-filled patch electrodes.

7. We compare the results with the data from multicellular tissue (Creed 1971). The more negative resting potential and the absence of spontaneous APs are mainly attributed to the absence of transmitter release from nerve terminals. The isolated cell is suggested as a model of the postsynaptic membrane properties.

Key words: Cell isolation – Smooth muscle cell – Action potential – Voltage clamp – Potassium current

Introduction

In recent years it has proved possible to disaggregate smooth muscle tissue enzymatically into individual cells (Bagby et al. 1971; Fay and Delise 1973; Momose and Gomi 1980; Benham and Bolton 1983). With slight modifications, we have utilized the collagenase dispersion technique of these reports to dissociate smooth muscle cells from the urinary bladder of the guinea-pig. We describe here the electrophysiology of the isolated cell in terms of the action potential (AP) and the net membrane currents as measured with intracellular (i.c.) electrodes and compare the properties of single cells with those published for multicellular bladder tissue. The comparison is essential in order to exclude that the proteolytic enzymes may have altered membrane proteins associated with the normal activity of "channels", "pumps", or "receptors".

Studying the ionic currents generating the AP, less artifacts are expected when a single cell is "clamped" than when a multicellular "strip" is used, because the latter consists of a multitude of elongated cells surrounded by a narrow extracellular (e.c.) "cleft" space, so that control of potential is compounded (e.g. see Bolton et al. 1981; Isenberg and Klöckner 1982b). Membrane currents from the smooth muscle of the urinary bladder have not been reported, therefore we shall show that the net current recorded from a single cell resembles the records published for uterine or intestine multicellular preparations; the obvious differences will be discussed in terms of series resistance as well as leakage and shunt currents. We shall compare the net and the potassium current in Results, so that the discussion can be concerned mainly with the similarities and differences between non- and dissociated bladder tissue. Furthermore, the particular problems when "patch pipettes" are applied to a cell of small volume are going to be discussed.

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Finally, we have attempted to analyse the net membrane current in terms of the individual components, i.e. the currents flowing through potassium or calcium channels. On the "whole cell" level, separation is usually performed by means of "channel blockers". In the present case however, we found that the Ca channel blockers also reduce the potassium outward currents, thus Ca inward current and activation of potassium current seemed to be linked (compare Walsh and Singer 1980b, 1981; Singer and Walsh 1984). Application of e.c. tetraethylammonium (TEA) blocked i_K only incompletely. Eventually, separation was achieved by blocking the potassium current with i.c. cesium ions. Problems dealing with the calcium inward current will be presented in the subsequent paper (Klöckner and Isenberg 1985). Preliminary reports of this work have been published previously (Klöckner and Isenberg 1984).

Methods

1. Cell isolation procedure. Guinea-pigs of either sex and of 400 g in weight were killed by cervical dislocation followed by decapitation. The urinary bladder was cut out and rinsed in a "calcium free medium" composed of 100 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM glucose, 50 mM taurine (adjusted with 5 mM MOPS/NaOH to pH 6.9). The pCa [$-\log$(calcium activity)], measured by means of a Ca electrode (Simon et al. 1978) was 6.0. The tissue was cut into chunks (2·2·2 mm) which were incubated for four periods of 30 min in the "enzyme medium" composed of 130 mM KOH, 20 mM taurine, 5 mM pyruvate, 5 mM creatine, adjusted with 10 mM HEPES and methansulfonic acid to pH 7.4 and complemented with 1 g/l collagenase (Sigma C 2139), 0.2 g/l pronase E (SERVA), 1 g/l fatty acid free albumin (FAFA). The pCa of the enzyme medium was adjusted to 4.2 by adding $CaCl_2$. The chunks were shaken at 35° C for 30 min, they were then reincubated in fresh enzyme medium. The cells were harvested in the supernatant. The greatest number of elongated cells was obtained with the incubation times of 90 and 120 min.

The cells could be stored at 5° C in a KB-medium ("Kraftbrühe") for periods of up to 1 week (compare Isenberg and Klöckner 1980, 1982a). The KB-medium was composed of 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 5 mM Na_2ATP, 5 mM K-pyruvate, 5 mM creatine, 20 mM taurine, 5 mM β-OH-butyrate, 1 g/l FAFA and adjusted with KOH to pH 7.2.

2. Experimental setup. The experimental chamber (2 mm by 2 mm by 12 mm) was placed on the stage of an inverted microscope adapted to a TV-camera system. Using a ZEISS LD 40 objective and an effective tubus length of 15 cm, the cell image on the 48 cm monitor had a final magnification of 2400 (see Isenberg 1982). A drop of KB-medium containing the isolated cells was pipetted into the chamber. When the cells had settled down and attached to the glass bottom (about 20 min), they were continuously superfused with a "physiological salt solution" (PSS) which was composed of 150 mM NaCl, 5.4 mM KCl, 3.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose (adjusted with 10 mM HEPES/NaOH to pH 7.4). The solution was prewarmed, so that the temperature measured close to the cells was 35° C. A complete change of the solution took about 20 s (as judged from the effects of 20 mM TEA).

3. Patch electrodes, electronics and data recording. The intracellular recordings were performed with patch electrodes ("whole cell recording", Marty and Neher 1983). Initially, the electrodes were pulled from haematocrit glass capillaries, later on from pyrex glass capillaries which gave less leakage current. After fire polishing, the electrodes were filled with an isotonic KCl-solution composed of 130 mM KCl plus 5 mM K-pyruvate, 5 mM K-oxalacetate, 5 mM K-succinate (for the reason of using these anions see Klöckner and Isenberg 1985). The pH of the solution was adjusted with 10 mM HEPES/KOH to 7.4 and the pCa with about 20 µM EGTA to 7.0. For comparison, some experiments were performed with electrodes containing no EGTA (pCa 6.0) or 1 mM EGTA, those results will be indicated in the text. In order to block potassium currents, in some of the experiments we filled the electrodes with a solution in which 130 mM CsCl substituted for the 130 mM KCl.

The electrodes had resistances of 3–4 MOhm and made electrical contact via a chlorided silver wire. A sintered Ag/AgCl pellet electrode grounded the bath. The input amplifier had the facility of a constant current source (10 nA/V, Dreyer and Peper 1974). The input current was adjusted to less than 10 pA. For the voltage clamp experiments the single electrode technique was used ("whole cell clamp technique" (Hamill et al. 1981). The voltage drop across the electrode resistance which is produced by the current flow was electronically compensated. The clamp worked with a settling time of less than 1 ms, and the voltage inhomogeneities remained below 3 mV (see Fig. 1 in Klöckner and Isenberg 1985).

Action potentials and membrane currents were digitized (10 bit resolution) and stored on tape (Macrodyn, ERDAC 4500); they were analyzed off-line with an interfaced Hewlett-Packard HP85 microcomputer. The figures were taken from the printouts of either the printer-plotter of the HP85 or of a HP 7225 paper plotter.

Results

1. Morphology

The enzymatic dissociation yielded elongated cells, partially contracted cells, round cells as well as cell debris (see Fig. 1). The elongated cells and the partially contracted cells ended in fine feet-like processes that spontaneously attached to the glass bottom of the chamber. Depolarizing clamp pulses usually triggered a shortening of the (unloaded) cell after a latency of about 0.4 s. Upon repolarization the cell relengthened, though most often, only incompletely. The elongated and the "partially contracted cells" did not differ in their electrophysiological properties. The round cells did not attach, and they showed neither APs nor calcium currents; they were not used for the analysis.

From 15 micrographs we evaluated the dimensions of the elongated cells (digitizing tableau MOP-AM 02). On the average, the cell length was 203 ± 23 µm (mean ± S.E.) and the cell diameter was 8.4 ± 0.7 µm. These values are very similar to the ones reported by Uvelius (1975) for the empty urinary bladder. We measured an average plate area of 711 ± 55 µm^2 and multiplying by 3.14 we obtained 2231 ± 179 µm^2 for the "external surface area A_s" (assuming a spindle-like cell). The cell volume, calculated by multiplying the plate area by the average diameter, amounted to 6281 ± 670 µm^3 (6.3 pl).

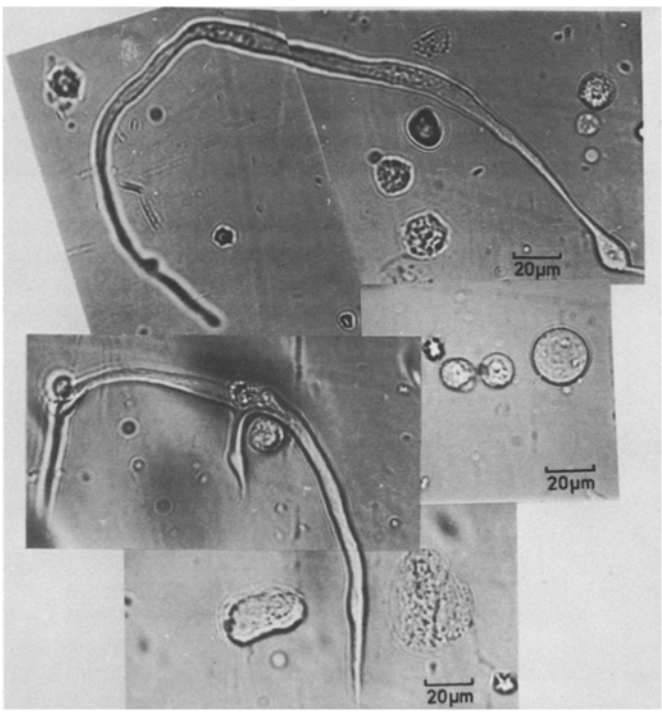

Fig. 1. Light microscopical photograph of the material released from the urinary bladder tissue by enzymatic dissociation. Cells of the elongated, of the partially contracted and the rounded type are shown together with debris. Note the feet-like processes. The figure was taken with a 40-fold water immersion objective in Nomarski-contrast

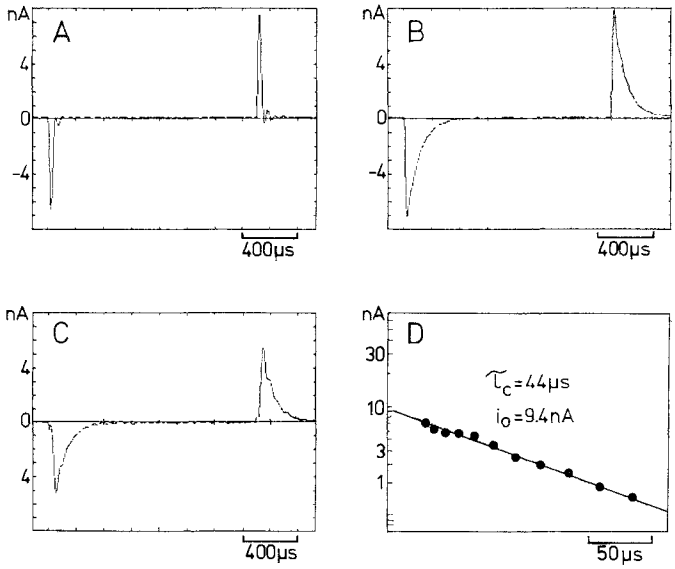

Fig. 2A–D. Capacitive currents as recorded with a single electrode voltage clamp from the isolated, elongated smooth muscle cell. The membrane is clamped from −65 to −75 mV. **A** Record from the patch of membrane; the capacitance can not be completely neutralized. **B** Record taken after having ruptured the patch of membrane. **C** Difference **C** minus **B**. **D** The decay of the capacitive difference current on semi-log coordinates

2. Resting potentials

In the constant current mode (200 ms pulses at 1 Hz) we pressed the tip of the electrode against the cell, build the Gigaohm seal by gentle suction and disrupted the membrane in the patch with a pulsative suction. Upon contact of the tip with the intracellular space the potential "jumped" by −45 and −55 mV. Mean resting potential of 35 cells was -52 ± 2 mV (±SE).

There was little or no change in the resting potential when we used electrodes filled with a KCl-solution without EGTA; the membrane potential averaged from 25 cells was -52 ± 3 mV. No clear-cut effects were seen when we changed to electrode solutions where the chloride was substituted by glutamate. However, the use of electrodes filled with Cs- instead of potassium salts depolarized the membrane. Two minutes after impalement the resting potential was $+9 \pm 5$ mV ($n = 9$).

3. Membrane capacitance, input- and series resistance

The membrane capacitance (c_m) was measured under voltage clamp conditions (electrodes filled with Cs-solution). In the cell-attached mode, we measured the component of capacitance between bath and electrode that could not be completely neutralized; after rupturing the membrane, the capacitance was measured again (Fig. 2). The difference in the capacitive currents was integrated (nA · s) and divided by the amplitude of the voltage step. From sixteen cells we obtained a mean value of $c_m = 50 \pm 6$ pF. This value was 2.2fold larger than the product surface area A_s times specific capacitance (1 µF/cm^2); the discrepancy might partially result from the fact that the light microscopy does not show areas of the infoldings or "pinocytotic vesicles" (Gabella 1981). The ratio of cell capacitance divided by 1 µF/cm^2 defines the "capacitive membrane area" A_c which will be used to transform the measured currents into current densities.

The capacitive transients rose within 20 µs to −5.2 nA and decayed exponentially (Fig. 2D). We used these transients to estimate the series resistance (R_s). Following the approximation of Connor et al. (1975) we obtained 960 ± 80 kOhm per cell or 48 ± 4 Ohm · cm^2. Using the method of Hodgkin et al. (1952) we estimated for R_s 880 ± 80 kOhm per cell or 44 ± 4 Ohm · cm^2. Some of the records showed capacitive transients which did not decay with a single but with two exponentials, the second time constant being in the order of 5 ms. We did not study this phenomenon since the square pulse method is inadequate for those details.

After settling of the capacitive transients, the amount of current flow is the sum of the leakage current and of current flowing over the input resistance of the cell. As long as we used electrodes pulled from haematocrit glass, 25 mV steps required currents of 26 ± 8 pA ($n = 12$). Using pyrex glass reduced this value to 6.5 ± 1 pA ($n = 10$). Provided that the leakage current can be neglected in the latter case, the input resistance is 3.8 ± 0.5 GOhm (electrodes filled with KCl-solution). Similar values of 4 ± 0.5 GOhm per cell ($n = 10$) were obtained from the slope of the $i-v$ curves between −25 and −75 mV (Fig. 11). Multiplication of the input resistance with the capacitive membrane area gives a value of 200 kOhm · cm^2. This value is considered to represent the specific membrane resistance since the membrane is clamped isopotentially (Fig. 1 in Klöckner and Isenberg 1985).

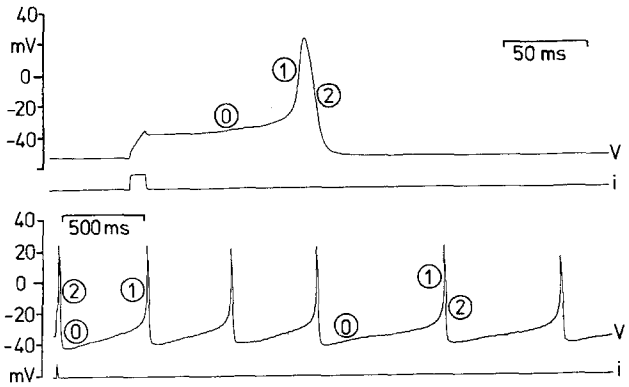

Fig. 3. Action potentials (APs). *Top:* a 9 ms long depolarizing current pulse (*trace i*) evoked a single AP (*trace V*). *Bottom:* a continuous cathodal current depolarized the resting membrane from −52 to −42 mV. At first, AP was triggered by a short pulse. Afterwards, the cell generates a train of APs. The numbers label the phases of slow depolarization (*0*), upstroke (*1*) and repolarization (*2*)

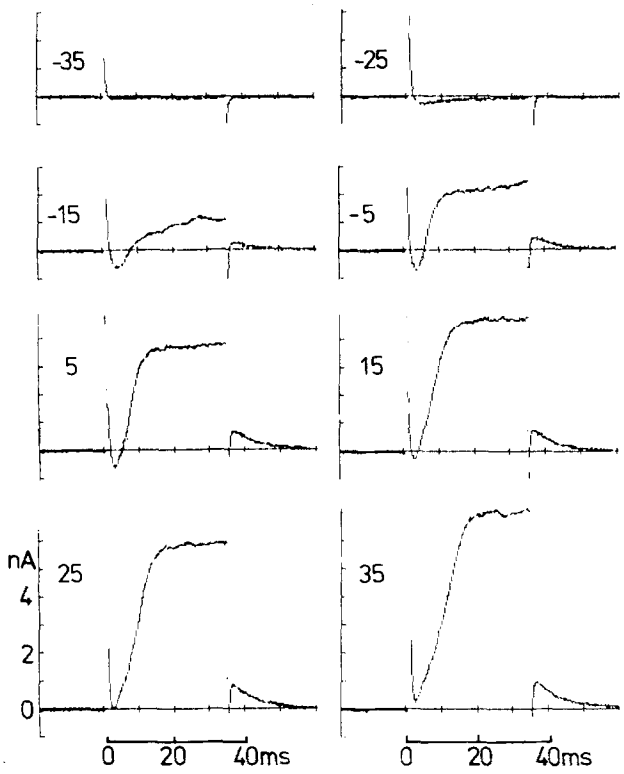

Fig. 4. Net membrane currents in response to 35 ms long clamp steps depolarizing from −55 to −25, −15 and +15 mV. Calibration valid for all traces

4. Action potentials

Smooth muscle cells of the urinary bladder are excitable, i.e. they respond to pulses of positive (cathodal) currents with action potentials (APs). In Fig. 3 (panel at the top) a current just above threshold depolarized the membrane from −52 to −38 mV. When the current was switched off, the potential did not recover but slowly turned more positive (phase 0, rate about 0.1 V/s). At about −30 mV, the rate of rise increased into the upstroke of the AP (labelled as phase 1, maximal rate of rise, $\dot{V}_{max}$, 6.5 ± 1.8 V/s, $n = 10$). The upstroke reached an overshoot of +22 ± 3 mV, thereafter, the membrane quickly repolarized back to the resting potential (phase 2 with $\dot{V}_{max}$ −6 ± 2 V/s). The duration of the AP, measured as the time between threshold and 90% repolarization (−40 mV), amounted to 31 ms (Fig. 2). Summarizing data from 20 cells, the AP amplitude was 71 ± 3 mV and the duration 36 ± 4 ms. Using electrodes filling with KCl but without EGTA, gave nearly the same results. Thus, the amplitude of the AP amounted to 73 ± 3 mV and the duration to 35 ± 5 ms ($n = 21$). Usually, the cells were not excited by anodal breaks. Anodal break APs were only recorded, if the cells had low resting potentials, e.g. due to leakage or due to block of potassium conductance with i.c. CsCl.

Continuous flow of cathodal current could induce repetitive firing. For Fig. 3 (bottom), the cell was depolarized with 5 pA to −35 mV. This depolarization by itself did not trigger an AP. An additional current pulse, however, initiated a first AP which was then followed by a train of "spontaneous" APs in the continuous presence of the cathodal current. The membrane repolarized from the first AP with an afterhyperpolarization (−47 mV), then, a "pacemaker depolarization" moved the membrane potential to the threshold (rate of 0.6 V/s), and a new AP started. Within the train, the rate of pacemaker depolarization slowed from AP to AP, and finally, after 5–10 APs, the excitations ceased altogether.

The upstroke of the AP is usually attributed to an inward current which is not carried by sodium but by calcium ions (urinary bladder: Creed 1971; vascular smooth muscle: Johansson and Somlyo 1980; toad stomach: Walsh and Singer 1980a). The following observations support this view: i) the upstroke remained unmodified when adding 30 μM tetrodotoxin to the bath or replacing sodium by tetramethylammonium. ii) $\dot{V}_{max}$ depended strongly on the e.c. calcium concentration; it decreased from 6.5 ± 1.8 to 3 ± 1.1 V/s when $[Ca]_o$ was reduced from 3.6 to 1.8 mM ($n = 5$), and it increased to 10.5 ± 4 V/s when $[Ca]_o$ was elevated to 10 mM ($n = 4$). iii) The excitability ceased when we added calcium channel blockers like 5 mM $NiCl_2$ or 0.5 μM D600.

5. Net membrane currents

The depolarization during the upstroke of an AP requires a net inward current, which should turn into a net outward current when repolarization occurs. The existence of a net inward current and the time course of its change is illustrated in Fig. 4. All clamp steps set between −35 mV (threshold of the AP) and +25 mV (overshoot of the AP) showed net inward currents. At −5 mV, the potential of $\dot{V}_{max}$ of the upstroke, the inward current peaked to −0.6 nA (−0.7 ± 0.1 nA mean from 15 cells). It changed into an outward current 12 ms after start of depolarization. The time until this change occurred decreased with more positive clamp steps, whereas the amount of outward current increased. After repolarization to −55 mV, a tail current followed which was outwardly directed and decayed with time.

The time dependence of i_m results from the superimposition of 2 current systems, the calcium inward current (i_{Ca}) and the potassium outward current (i_K). i_{Ca} generates the negative current surge and thereby the phases 0 and 1 of the AP; details of it are presented in the following paper (Klöckner and Isenberg 1985). Here we shall analyze i_K.

Because i_m is a net current, we should have "dissected" i_K by blocking i_{Ca}. However, all attempts with Ca channel blockers (0.5 µM D600 or 5 mM Ni, see Fig. 5) simultaneously reduced i_K, a result which favours the idea that i_K is activated by an increase $[Ca]_i$ (details in preparation).

6. *The tail current*

The conductances which have been activated by a depolarizing pulse, "deactivate" upon repolarization and this produces a tail current. We evaluated the tail current for times later than 2 ms after repolarization; at these later times, the superimposed Ca tail current has already deactivated (see Fig. 1 in Klöckner and Isenberg 1985) and the amplitude of the tail current is now a good indicator of the potassium conductance g_K.The amplitude of the tail current depends on the actual potential V_m (−55 mV in Fig. 4) where the difference between the potassium equilibrium potential E_K and V_m determines the driving force (V_m-E_K). The dependence of the tail current on (V_m-E_K) shows a reversal potential (E_{rev}) at −85 mV (flat trace in Fig. 6A). For prepulses longer than 2 ms, the prepulse duration did not modify E_{rev}. E_{rev} observed in 4 cells was -82 ± 2 mV. E_K calculated from $[K]_o = 5.4$ mM and $[K]_i = 150$ mM is −88 mV. Since E_{rev} was close to E_K, it is suggested that the tail current is predominantly carried by potassium ions.

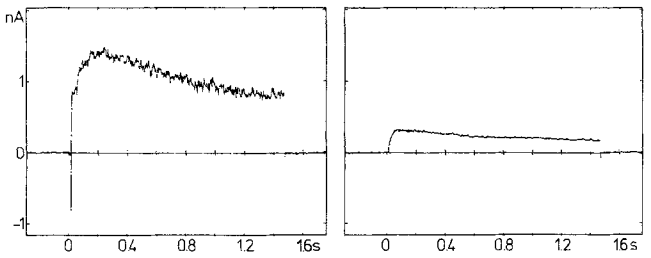

Fig. 5. The Ca-channel blocker nickel reduces the outward currents. 1.45 s long pulses from −55 to +5 mV were applied before and after addition of 5 mM $NiCl_2$ to the PSS (*left* and *right frame*, respectively)

The prepulse potentials modified the amplitude of the tail current in a "bell-shaped" manner similar to the outward current during the prepulse (Fig. 6B). This result suggests that the tail current reflects the decay of a potassium conductance which was activated during the depolarization.

Figure 6C shows the increase in the amplitude of the tail current for varied periods of depolarization. Up to 200 ms, the amplitude increased with the length of the pulse. When longer prepulses inactivated the potassium conductance, the amplitude fell to very low levels (Fig. 6D, arrow). From the envelope of the amplitudes of the tail current, one can construct the activation time course of the outward current (line through the circles in Fig. 6C). Activation of i_K as evaluated from the tail currents lies above the net current during the prepulse as expected if i_{Ca} superimposes on the current during the prepulse but not on the envelope of the tail current (see above).

The decay of the tail current followed a single exponential which probably represents the voltage-dependent turning-off of activation. The time constants were 10 ms at −10 mV, 5 ms at the resting potential of −50 mV, and 1−2 ms at −100 mV. Such a time course is fast in comparison to the activation and the inactivation time course during depolarization.

7. *Voltage dependence of the outward current*

During long depolarizing clamp steps, the outward currents increased to a peak which was reached after 100−300 ms (Fig. 7A). Afterwards, the outward currents slowly decayed.

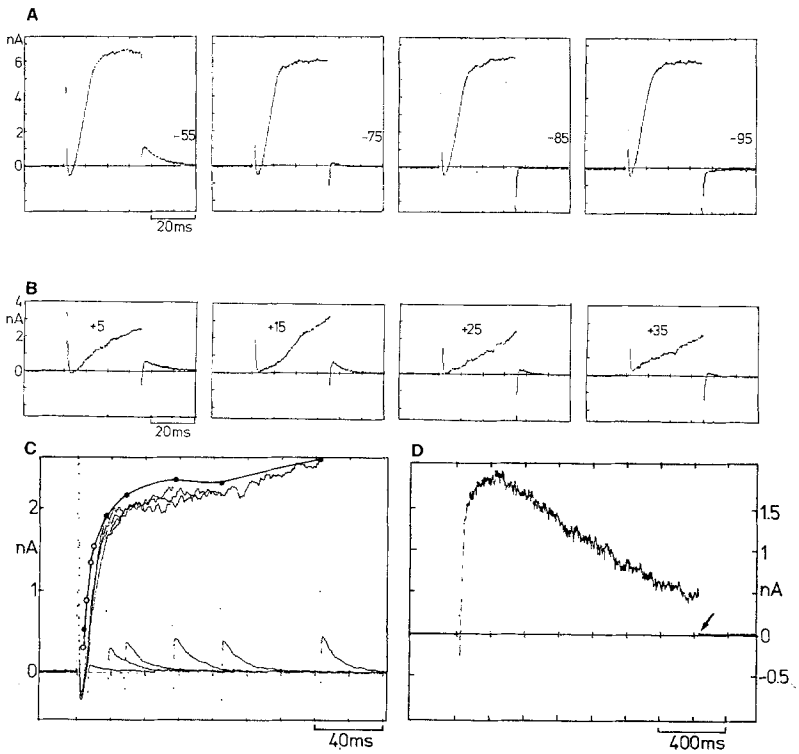

Fig. 6A−D
Analysis of the tail current (different cells). **A** Evaluation on the reversal potential. 36 ms long prepulses to −5 mV were followed by repolarizations to the indicated potentials. Note: the reversal potential is indicated by the horizontal tracing at −85 mV. **B** The amplitude of the tail current varies similar to the amount of i_K activated by the prestep (potentials indicated, tail current at repolarization to −43 mV). **C** The amplitude of the tail current increases when i_K activates during the prepulse. The curve through the circles indicates the activation time course of i_K as evaluated from the envelope of the tail currents (amplitude normalized to peak i_K, empty circles from measurements with faster sweep speed). Prestep to 0 mV, repolarization to −55 mV. **D** Inactivation of i_K has strongly diminuished the amplitude of the tail current (*arrow*)

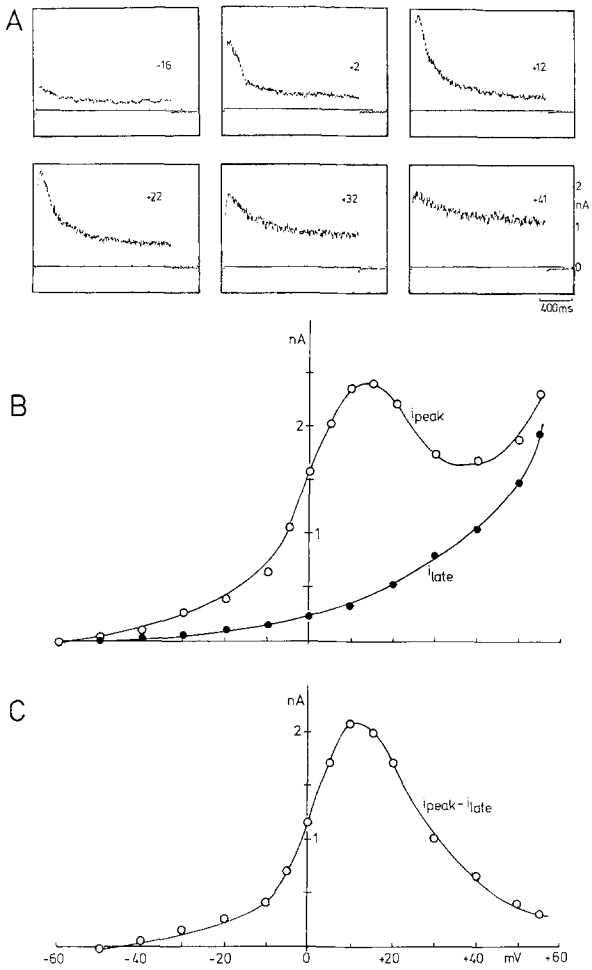

Fig. 7A–C. Influence of the clamp step potential on the outward current. Holding potential −65 mV, pulse length 1.7 s. **A** Net currents, pulse potential indicated. **B** Current-voltage relations of the positive peak current and the late current at the end of pulse. **C** Current-voltage relation of the difference i_{peak} minus i_{late}

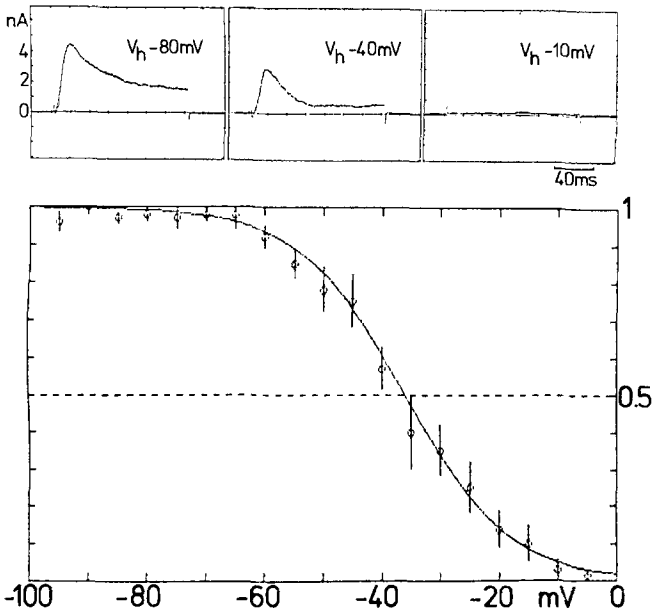

Fig. 8. Steady-state inactivation curve of the peak outward current. *Insets:* when the holding potential (V_h) was set for 5 s to more positive values, the outward current became depressed (140 ms long steps to −15 mV). *Bottom*: the normalized currents (*ordinate*) were plotted as a function of holding potential (*abscissa*). Normalization was performed to bring the maximal current to 1 (usually current for V_h more negative than −80 mV). The data indicated the mean ± SE from six cells. The curve was calculated with $\{1 + \exp[(V + 36\text{ mV})/9\text{ mV}]\}^{-1}$

We call the decay inactivation without judging whether this process depends primarily or voltage of $[Ca]_i$. During the inactivation, the net current should approximate i_K reasonably well since the interfering i_{Ca} has inactivated to less than 0.2 nA (Klöckner and Isenberg 1985).

Generally, the inactivation time course was slower the more positive the clamp step potential was set. Occasionally, we were able to fit the inactivation with two exponentials (for example in Fig. 5, +22 mV, 80 ms and 920 ms, respectively), but more often this was not possible.

The voltage-dependence of the outward current was evaluated with the current-voltage relations ($i-v$ curves) of Fig. 7, where the peak current and the current at the end of the 1.7 s long pulses were plotted (i_{peak} and i_{late}, respectively). Between −70 mV and −20 mV, the peak was missing and i_{late} was smaller than 10 pA. Positive to −20 mV, i_{late} increased in an outwardly rectifying voltage dependence (dots in Fig. 7B). Correspondingly, the slope conductance increased from 0.34 nS at −50 mV over 10 nS at 0 mV to 42 nS at +50 mV. Outward rectification of i_{late} was observed in all 12 cells investigated confirming the results from single toad stomach cells (Singer and Walsh 1980 b; Walsh and Singer 1981) as well as from multicellular preparations of the uterus (Anderson 1969; Mironneau 1974; Kao and McCullough 1975) or the *Taenia coli* (Inomata and Kao 1976).

The $i-v$ curve of the peak current followed an N-shaped curve. The curve had a threshold at −30 mV and increased steeply with more positive potentials. It reached a relative maximum at +15 mV from which it fell again (circles in Fig. 7B). The curve of the difference current i_{peak} minus i_{late} was bell-shaped (Fig. 7C). In the experiment of Fig. 7 maximal current was seen at +12 mV, but in other cells this maximum was found at more positive potentials ($+36 \pm 6$ mV, $n = 12$). N-shaped $i-v$ curves of the peak outward current have been reported for isolated toad stomach cells (Walsh and Singer 1981) as well as for multicellular uterine preparations (Vassort 1975). The bell-shape of the difference currents resembles the mirror image of the $i-v$ curve of peak i_{Ca} (Klöckner and Isenberg 1985), it has been suggested that this similarity is evidence for time-dependent current being activated by $[Ca]_i$ (Meech 1978). This view is supported by recent single channel recordings (Benham et al. 1985; Singer and Walsh 1984).

Steady-state inactivation of i_K. Holding potentials more positive than the resting potential attenuated i_{peak} and i_{late}, and those more negative facilitated them (Fig. 8, inset). Results from 10 cells (mean ± SE) are summarized in the steady-state inactivation curve of i_{peak} (Fig. 8, bottom). The curve was calculated according to

$$h_\infty = \{1 + \exp[(V_m - V_{0.5})/k]\}^{-1}$$

where according to a least square fit, the slope (k) was 9 mV and the potential of half maximal inactivation ($V_{0.5}$) was

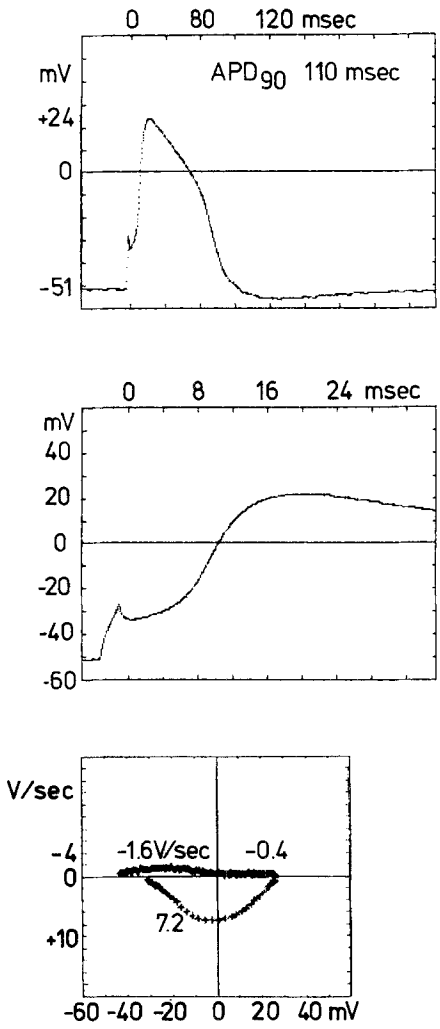

Fig. 9. Action potentials recorded in PSS containing 20 mM tetraethylammonium chloride (TEA). The *panel* in the *middle* shows the upstroke with an expanded time scale. The *plot* at the *bottom* is a "phase plane" plotting the derivative of the potential (digital differentiation) as a function of the actual membrane potential. The numbers lable $\dot{V}_{max}$ for the upstroke and the two parts of repolarization

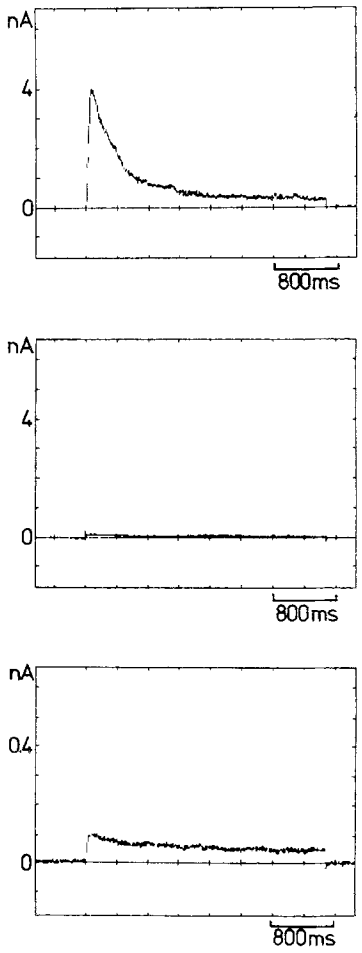

Fig. 10. 20 mM tetraethylammonium chloride (TEA) reduce but do not block i_K. 3.4 s long pulses from −65 to +30 mV. *Top:* Before, middle 20 s after application. *Bottom:* 25 s after application but recorded with 10-fold higher gain

−36 mV. The steady state inactivation curve reveals that at the resting potential of −50 mV 80% of the outward current is available (80%), and that holding the potential at −65 mV brings the availability close 100%.

8. Action potentials and membrane currents in presence of 20 mM TEA

TEA is known to reduce the potassium permeability in a variety of different smooth muscle preparations (Vassort 1975; Singer and Walsh 1980b; Imaizumi and Watanabe 1981). As expected from these effects, urinary bladder cells responded to the addition of 20 mM TEA with a prolongation of the AP duration which increased within 20 s from 32 ± 3 ms (control) to 210 ± 20 ms ($n = 8$). The prolongation resulted mainly from a retarded repolarization positive to −10 mV. Since the rate of repolarization speeded up at more negative potentials, a "trapezoidal" shape of the AP resulted (Fig. 9). Regarding the upstroke of the AP (phase 1), TEA increased its rate of rise and the overshoot. The TEA-effects on the resting membrane potential were insignificant ($P = 0.1$, $n = 8$). When present, pacemaker depolarizations continued in the presence of TEA. TEA did not induce spontaneous activity.

Membrane currents. 20 mM TEA largely reduced but did not block i_K; e.g. at 30 mV, the peak of net outward current fell from 4 to 0.2 nA and the steady-state current from 0.4 to 0.1 nA (Fig. 10). The peak inward current (due to i_{Ca}) became more pronounced and remained negative for a longer period of time. The current, however, did become outward. The late outward current followed an N-shaped voltage dependence. Also, TEA did not block the tail currents. The block of i_K remained incomplete even when the cells were superfused with a medium in which all the 150 mM NaCl had been substituted by TEA-Cl. Thus, 20 mM TEA can not be used as a "tool" to dissect i_{Ca} from i_K.

In orienting experiments, 5 mM 4-aminopyridine ($n = 5$) or 5 mM 4-aminoacridine ($n = 2$) did not affect i_K. It is concluded that a 4-aminopyridine-sensitive i_K does not exist in the smooth muscle cell of the urinary bladder.

9. Block of i_K by i.c. cesium

Patch electrodes have a relatively large opening through which the electrolyte, filling the electrode, may diffuse into the cell. If the cell volume is small, the filling electrolyte should rapidly equilibrate with the i.c. space of the cell (compare Fenwick et al. 1982). Making use of this phenomenon, we tried to block i_K applying electrodes filled with 150 mM TEA-Cl. The resulting reduction of i_K was rather small (periods up to 15 min; Fig. 11, $i-v$ curve through the x-es). (A similar little blocking action of internal TEA has been reported for the Ca-activated i_K of Aplysia neurons by Hermann and Corman 1981).

Unlike i.c. TEA, however, the use of Cs-filled electrodes blocked i_K within 2 min. (The time course for the onset of the block is described in Fig. 2 of the following paper Klöckner and Isenberg 1985.) The "Cs-loaded cells" depolarized to potentials between 0 and +20 mV, therefore, the cells had to be stabilized by voltage clamp. Plotting the late current against voltage (circles in Fig. 11) resulted in an $i-v$ curve that was straight and flat from −95 mV up to +30 mV. The slope conductance of 0.04 nS corresponded to an input resistance of 24 GOhm (difficult to distinguish from the seal resistance). At potentials positive to +30 mV the late outward current increased which might be attributed either to the removal of the Cs-block by the large positive driving force and/or to outward currents carried by monovalent cations through Ca-channels (Tsien 1983).

The block of i_K by i.c. Cs was attenuated by the simultaneous presence of TEA; though the outward currents were smaller than those recorded with KCl-electrodes they were larger than the currents recorded with Cs-electrodes and followed the typical N-shape (Fig. 11, crosses, electrode filled with a mixture of 100 mM CsCl plus 50 mM TEA-Cl, similar results in four other cells).

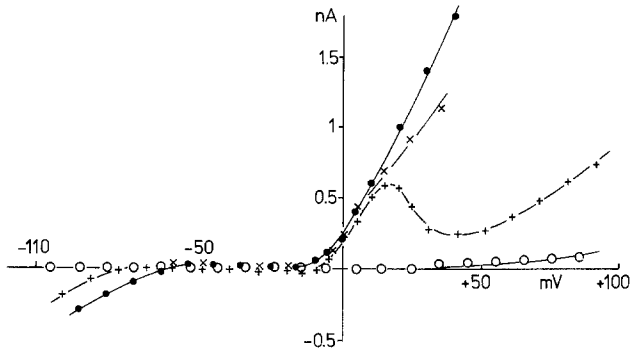

Fig. 11. i_K as modified by i.c. applied TEA or Cs. The $i-v$ curves were measured in different cells of comparable membrane capacitance. The curves were obtained with electrodes filled with KCl-solution (●), a solution where all potassium was replaced by TEA (x-es), the Cs-solution (○), and a 2:1 mixture of the Cs- and TEA-solution (+)

Discussion

In this paper, we have introduced the smooth muscle cell isolated from the urinary bladder of the guinea-pig as a new preparation suitable for electrophysiological experiments. Regarding the work with isolated cells, we followed a trend that started in 1971 with the pionieering work of Bagby and coworkers (1971, 1973), of Fay and Delise (1973) and later by Walsh and Singer (1980a, b; Singer and Walsh 1980). Whereas all these papers delt with the stomach of the toad, we isolated cells from the mammalian urinary bladder. Since both preparations deal with isolated visceral smooth muscle cells, we expected to find some common basic electrophysiological properties but differences in the detail. The cells originate from a different organ of different species and the experimental conditions differ (35° C, 3.6 mM $CaCl_2$ versus room temperature and 15−50 mM $CaCl_2$ in Walsh and Singer 1980a, 1981).

In spite of following the literature using collagenase as the main enzymatic principle (Bagby et al. 1971) and making use of our own experience in isolating mammalian ventricular cells (Isenberg and Klöckner 1980, 1982a; Bendukidze et al. 1985), we needed half a year to optimize the procedure by trial and error. Our criteria were based both on morphology and electrophysiology: the shape of the dissociated cells should be elongated and not round, and the cells should be excitable (which requires not only reasonable resting potentials but also calcium currents). With these criteria, we found that the two enzymes collagenase and pronase provided the best results. Arguing that the cells gain sodium and lose potassium during the dissociation, we used a medium with high potassium and low sodium content (compare Hermsmeyer and Mason 1982). The advantage of replacing the chloride by methansulfonate was found by trial and error. (We prefer methansulfonate to glutamate, as suggested by Hermsmeyer and Mason 1982, for glutamate binds calcium.)

In the light microscope the isolated elongated cells resembled the structures seen in the non-dissociated urinary bladder tissue (Uvelius 1975; Gabella 1981) and they have about the same dimensions as the cells isolated from the toad stomach (compare our data on p. 330 with Singer and Walsh 1980). From the outer dimension we calculated a cell volume of 6.3 pl; it is assumed that this small volume will rapidly equilibrate with the solution diffusing out of the mouth of the patch electrode. This diffusional contact is of advantage when we whish to apply substances to the i.c. space as we did with CsCl, however, it also raises problems. With regard to the osmolarity, the "filling electrolyte" has to be "isotonic" with the e.c. salt solution. Filling the electrodes with 0.4 M KCl solution induced "blebbing" of the cell membrane within 1 min.

Even when isotonic, the filling electrolyte is expected to disturb the i.c. ionic composition. To our surprise, obvious electrophysiological changes were rare. Thus, neither resting and action potentials nor potassium currents changed when we used filling solutions without EGTA though these solutions were contaminated with about 5 μM Ca. (A small effect on i_{Ca} will be reported in the following paper, Klöckner and Isenberg 1985.) Since elevated $[Ca]_i$ is supposed to activate i_K, the absence of measurable effects suggests that the cell can handle the extra Ca-load without any problems. Electrodes filled with KCl or K-glutamate, also did not influence the electrophysiological parameters. Again we interpreted that either the cell can maintain a constant cytosolic chloride concentration despite of the diffusion from the electrode or that changes in $[Cl]_i$ are not sensitively reflected by our electrophysiological data.

The main purpose of isolating the cells was to obtain a preparation which was thought to be better suited for the

voltage clamp experiments than multicellular "strips" are. Since this technique "clamps" the membrane by a feedback supply of current which produces a voltage drop over the "resistance" of the membrane, its validity requires that the voltage drop originates exclusively from the membrane. The requirement of negligible series resistances (R_s) seems to be fullfilled in the present case, where R_s between the cell membrane and the bath amounted to 44 Ohm · cm^2. It is about 10-fold lower than the 550 Ohm · cm^2 reported by Kao and McCullouch (1975) for the non-distributed part of R_s in multicellular preparations. Resistance in the e.c. cleft space could add an additional 1 kOhm · cm^2 and prevent adequate voltage control when currents are fast and of large amplitude (Johnson and Lieberman 1971; for a detailed discussion see also Isenberg and Klöckner 1982b). The single electrode clamp technique raises a special problem for the electrode resistance is in series with the membrane; but, this series resistance can be electronically compensated (Dreyer and Peper 1974) and we shall show that artifacts can be avoided as long as the electrode resistance is below 4 MOhm (Klöckner and Isenberg 1985).

Extrapolating from the single cell model to the in vivo situation is tempting, but requires caution. The proteolytic enzymes collagenase and pronase may have altered membrane proteins which are linked to the functions of "channels" or "receptors". Therefore, it is essential to compare the membrane resting and action potentials of isolated cells with those reported for the multicellular perparations in the literature. Resting potentials of −37 mV have been reported for strips from the urinary bladder (Creed 1971; Creed et al. 1983; Kurihara and Creed 1972), this value is significantly less negative than the value of −52 mV reported here. We discuss the discrepancy as follows: a) the microelectrode may be impaled with a leakage which "shunts" the true potential. With the isolated cell we discard the experiment when leakage is suggested by a low resting potential and low input resistance. In multicellular tissue the damage might not have been discovered since the neighbouring cells, which contribute to the input resistance as well, may have "held" the potential at a value which is only slightly diminished. On the other hand, cell damage normally produces not a de- but a hyperpolarization for a transient period of time ("penetration induced hyperpolarization", Walsh and Singer 1980b). Thus, we consider this explanation to be unlikely. b) Isolated cells rest unstretched on the floor of the chamber whereas the multicellular strips are stretched in some degree, and stretch is supposed to depolarize the membrane (Johansson and Mellander 1975). c) In the strip, a steady release of neurotransmitters (acetylcholine) depolarizes the "true" resting potential (Creed et al. 1983; Sibley 1984). The isolated cell is deprived of nerve terminals, therefore the modulating transmitter action is missing. (The influence of "presynaptic effects" via nerve terminals is even stronger when the multicellular preparation is field stimulated, see e.g. Krell et al. 1981; Creed et al. 1983; Sibley 1984.)

The isolated cells rarely fire APs spontaneously, usually they generate APs only in response to (electrical) stimulation. Strips from the urinary bladder are usually spontaneously active (Anderson et al. 1972), they stop their activity only when the resting potentials is more negative than −45 mV or when $[Ca]_o$ is elevated from 2.5 to 7.5 mM (Creed 1971). In the isolated cells, repetitive firing could be induced by depolarizing the membrane with sustained cathodal currents. Since the absence of repetitive activity and more negative resting potentials go hand in hand, the factors discussed above for the difference in resting potential could also account for the difference in repetitive firing. The individual spike in the single cell rose from a resting potential of −52 mV with a $\dot{V}_{max}$ of 6.5 V/s to an overshoot of +20 mV. For the multicellular strip with a resting potential of −37 mV, $\dot{V}_{max}$ was 4.2 V/s and the overshoot +12 mV (Creed 1971). Depolarizing the single cell to −37 mV attenuated $\dot{V}_{max}$ and overshoot in such a way (Fig. 2B) that the numbers became identical with those reported for the strip. We sum up in concluding that the APs have no peculiar property that cannot be attributed to the less negative resting membrane potential of the multicellular preparation.

The net membrane current seems to be composed mainly of i_K and i_{Ca}. "Pharmacological" separation of these components, e.g. isolation of i_K by blocking i_{Ca}, did not work because the Ca channel blockers simultaneously reduced i_K. The isolation of i_{Ca} with 20 mM TEA did not work either, since i_K was only reduced but not blocked; our results suggest that the current measured in the presence of 20 mM TEA describes neither the time course nor the voltage dependence of i_{Ca} adequately. (Such an approximation was used e.g. by Vassort 1975; Inomata and Kao 1976.) With the isolated cell we can effectively block i_K by i.c. CsCl, i_{Ca} which is left in those "Cs-loaded" cells will be analysed in the following paper (Klöckner and Isenberg 1985).

We concluded that the outward currents were potassium currents. Our evidence was based on a) the reversal potential of the tail current and b) the sensitivity of the current to e.c. TEA and i.c. Cs. This potassium current may flow not only through one but through several types of potassium channels, a first which is insensitive to potential but activated by an increase in $[Ca]_i$ (i_{KCa}) and a second one which is activated by membrane depolarization as it was suggested by single channel analysis (Benham et al. 1983; Benham et al. 1985; Singer and Walsh 1984; Berger et al. 1984). We intend to attribute peak i_K to i_{KCa}: it is blocked by Ca-channel blockers, its N-shaped voltage dependence parallels the one of i_{Ca} (Isenberg and Klöckner 1985), and the availability curve of i_K resembles that of i_{Ca}. However, a more careful comparison reveals that parameters of these curves differ. The potential of half maximal activation was +2 mV for i_K and −14 mV for i_{Ca} and for inactivation −36 mV (i_K) have to be compared with −43 mV (i_{Ca}). Also, the slope factors of the availability curves differ (9 mV for i_K and 6 mV for i_{Ca}). Ca entering with i_{Ca} binds not only to sites activating i_K and the Ca, activating i_K, can derive not only from i_{Ca} but also from intracellular stores. The aequorin measurements in isolated toad stomach cells (Fay et al. 1979) indicate that $[Ca]_i$ slowly decays during sustained depolarization, the decay of $[Ca]_i$ may account for the "inactivation" of i_K. On the other hand, the $[Ca]_i$ dissappears slowly also during repolarization whereas the tail currents decayed with a time constant of several ms. Thus, repolarization may close Ca-activated channels in a primarily voltage-dependent manner.

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