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# Synchronized $\text{Ca}^{2+}$ signaling by intercellular propagation of $\text{Ca}^{2+}$ action potentials in NRK fibroblasts

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**De Roos, Albert D. G., Peter H. G. M. Willems, Everardus J. J. van Zoelen, and Alexander P. R. Theuvenet.** Synchronized  $\text{Ca}^{2+}$  signaling by intercellular propagation of  $\text{Ca}^{2+}$  action potentials in NRK fibroblasts. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1900–C1907, 1997.—The intercellular propagation of  $\text{Ca}^{2+}$  waves by diffusion of inositol trisphosphate has been shown to be a general mechanism by which nonexcitable cells communicate. Here, we show that monolayers of normal rat kidney (NRK) fibroblasts behave like a typical excitable tissue. In confluent monolayers of these cells,  $\text{Ca}^{2+}$  action potentials can be generated by local depolarization of the monolayer on treatment with either bradykinin or an elevation of the extracellular  $\text{K}^+$  concentration. These electrotonically propagating action potentials travel intercellularly over long distances in an all-or-none fashion at a speed of  $\sim 6.1$  mm/s and can be blocked by L-type  $\text{Ca}^{2+}$  channel blockers. The action potentials are generated by depolarizations beyond the threshold value for L-type  $\text{Ca}^{2+}$  channels of about  $-15$  mV. The result of these locally induced, propagating  $\text{Ca}^{2+}$  action potentials is an almost synchronous, transient increase in the intracellular  $\text{Ca}^{2+}$  concentration in large numbers of cells. These data show that electrically coupled fibroblasts can form an excitable syncytium, and they elucidate a novel mechanism of intercellular  $\text{Ca}^{2+}$  signaling in these cells that may coordinate synchronized multicellular responses to local stimuli.

bradykinin; intracellular calcium; calcium channels; normal rat kidney fibroblasts

INTERCELLULAR COMMUNICATION plays an important role in the coordination of cooperative cellular responses. In excitable cells, such as nerve and muscle cells, action potentials caused by the regenerative opening of voltage-dependent channels provide a fast (0.1–100 m/s) mechanism for signaling over long distances. In nonexcitable cells, coupling via gap junctions provides a way for intercellular signaling, and it has been shown that the intercellular diffusion of D-*myo*-inositol 1,4,5-trisphosphate [ $\text{Ins}(1,4,5)\text{P}_3$ ] can induce  $\text{Ca}^{2+}$  waves that slowly propagate (20–50  $\mu\text{m/s}$ ) from cell to cell (26).

Fibroblasts are considered to be a classic example of nonexcitable cells. Fibroblasts can be electrically coupled to each other (7, 15, 18, 25), and in several tissues, such as kidney, intestine, and dermis, these cells form three-dimensional communicating networks (19). Fibroblasts can also be electrically coupled to other cells, including myocytes (24) and endothelial (17) and neuronal (6) cells. Electrical coupling via gap junctions provides a way for the fast transduction of membrane potential changes between cells and can thus coordinate signaling. Although it has been reported that fibroblasts possess voltage-dependent  $\text{Ca}^{2+}$

channels (3, 5, 13, 21, 22) that would in principle enable these cells to generate action potentials, a function for such channels in these cells has so far been unclear.

Monolayers of normal rat kidney (NRK) fibroblasts are electrically well coupled (7) and therefore provide a model system to investigate the intercellular transduction of electrical signals. NRK fibroblasts, clone 49F, can be made quiescent by serum deprivation of confluent cultures. When these quiescent cells are subsequently treated with epidermal growth factor (EGF) as the only growth-stimulating polypeptide, the cells undergo one additional cell cycle before they undergo density-dependent growth inhibition or density arrest (29). We recently showed that spontaneous repetitive membrane potential depolarizations were observed in  $\sim 50\%$  of monolayer cultures of density-arrested NRK cells (9). Although these depolarizations showed the characteristics of action potentials, the spontaneous occurrence of the depolarizations in these cells did not allow a further study on the mechanism of propagation and induction of action potentials. In contrast to the density-arrested cells, quiescent NRK fibroblasts never exhibited spontaneous action potential-like depolarizations.

In the current study, we show that in quiescent NRK cells action potentials can be induced by bradykinin (BK), which allowed the study of the mechanism of propagation and induction of action potentials in these cells. BK is an agonist that not only increases intracellular  $\text{Ca}^{2+}$  (1) but also depolarizes NRK fibroblasts via an increased  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance (8). In monolayers of quiescent NRK fibroblasts, local depolarization of only a small part of the monolayer with BK can induce an intercellularly propagating  $\text{Ca}^{2+}$  action potential that results in a fast transient increase in the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in large numbers of cells. The  $\text{Ca}^{2+}$  action potentials reported here provide a mechanism for intercellular communication that is several magnitudes faster than the reported intercellular  $\text{Ins}(1,4,5)\text{P}_3$ -mediated  $\text{Ca}^{2+}$  waves in nonexcitable cells.

## EXPERIMENTAL PROCEDURES

NRK cells (clone 49F) were seeded at a density of  $1.0 \times 10^4$  cells/cm<sup>2</sup> and grown to confluence in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum as previously described (1). The tissue culture dishes that were used had diameters of 35, 55, 85, and 140 mm, containing 2, 4, 10 and 20 ml of media, respectively. Confluent cultures were incubated in serum-free DF medium (1:1 DMEM-Ham's F-12) supplemented with 30 nM  $\text{Na}_2\text{SeO}_3$  and 10  $\mu\text{g/ml}$  human transferrin for 2–4 days, by

which time the cells became quiescent. DF medium is a cell culture medium that contains as main inorganic salts (in mM) 109.5 NaCl, 5.4 KCl, 1.8  $\text{CaCl}_2$ , 0.81  $\text{MgCl}_2$ , 44.0  $\text{NaHCO}_3$ , and 1.0  $\text{NaH}_2\text{PO}_4$  and is supplemented with essential nutrients such as glucose, amino acids, and vitamins for optimal cell growth. For patch-clamp experiments, cells were incubated in either normal  $\text{Ca}^{2+}$ -containing DF medium or nominally  $\text{Ca}^{2+}$ -free DF medium supplemented with the indicated concentrations of  $\text{SrCl}_2$  and equilibrated with 5%  $\text{CO}_2$  to a pH of 7.4. When  $\text{SrCl}_2$  was added to the medium, the concentration of NaCl was lowered to maintain osmolarity. When ion substitutions were made, *N*-methyl-D-glucamine was substituted for  $\text{Na}^+$ , whereas gluconate was substituted for  $\text{Cl}^-$ . 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) loading was achieved by incubating the cells for 30 min in the presence of 100  $\mu\text{M}$  BAPTA-acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR), after which the cells were incubated in DF medium for 10 min.

Whole cell patch-clamp measurements were carried out as previously described (7). Patch pipettes were made from thin-walled glass (SG150T, Clarke Electromedical Instruments, Pangbourne, UK) using a two-stage pipette puller (L/M-3P-A, List Electronic, Darmstadt, Germany). Pipettes were filled with a solution containing (in mM) 25 NaCl, 120 KCl, 1  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 3.5 ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10 tris(hydroxymethyl)aminomethane (pH 7.4) and had resistances of 4–6 M $\Omega$ . Membrane potential was detected with an EPC-7 patch-clamp amplifier (List Electronic) in the current-clamp mode. Because monolayers of NRK cells are electrically well coupled, there is electrical access from the patched cell to neighboring cells (7). Therefore, the measured membrane potential will be an average of many coupled cells, and only the intracellular components of the patched cell are washed out. In this way, stable resting membrane potentials could be measured for >1 h.

Ratio fluorometry for measurements of  $[\text{Ca}^{2+}]_i$  was performed as previously described (31). In short, cells were loaded with 1.5  $\mu\text{M}$  fura 2-AM (Molecular Probes) for 30 min and washed for 20 min in DF medium. Excitation wavelengths were 340 and 380 nm, and emission was monitored at 492 nm. Dynamic video imaging was carried out using the MagiCal hardware and Tardis software of Joyce Loebel (Tyne and Wear, UK).  $[\text{Ca}^{2+}]_i$  were calculated after calibration of fluorescence ratios to free  $[\text{Ca}^{2+}]_i$  from five individual experiments using 10  $\mu\text{M}$  ionomycin and 20 mM EGTA to obtain maximal and minimal  $[\text{Ca}^{2+}]_i$ , respectively.

Figure 1A shows the experimental setup for most of the patch-clamp experiments. A cell from a monolayer culture was patched, and a depolarizing stimulus was given by the manual application of a small volume (5 or 10  $\mu\text{l}$ ) of 10 nM BK or 124 mM KCl at a certain distance from the measuring patch-clamp pipette (distant stimulus) using a 20- $\mu\text{l}$  Gilson micropipette. By this mode of application, it was ensured that only a small part of the monolayer was exposed to stimulatory concentrations of the stimulus and that the membrane potential could be recorded in cells that were not directly exposed to the stimulus. Also, propagation of membrane potential signals was measured by using two patch-clamp pipettes that were located at a certain distance (700–900  $\mu\text{m}$ ) from each other (Fig. 1B). In this case, propagation of a signal could be determined from the delay in response between the two pipettes when a distant stimulus, similar to the one in Fig. 1A, was given on either side of each pipette.

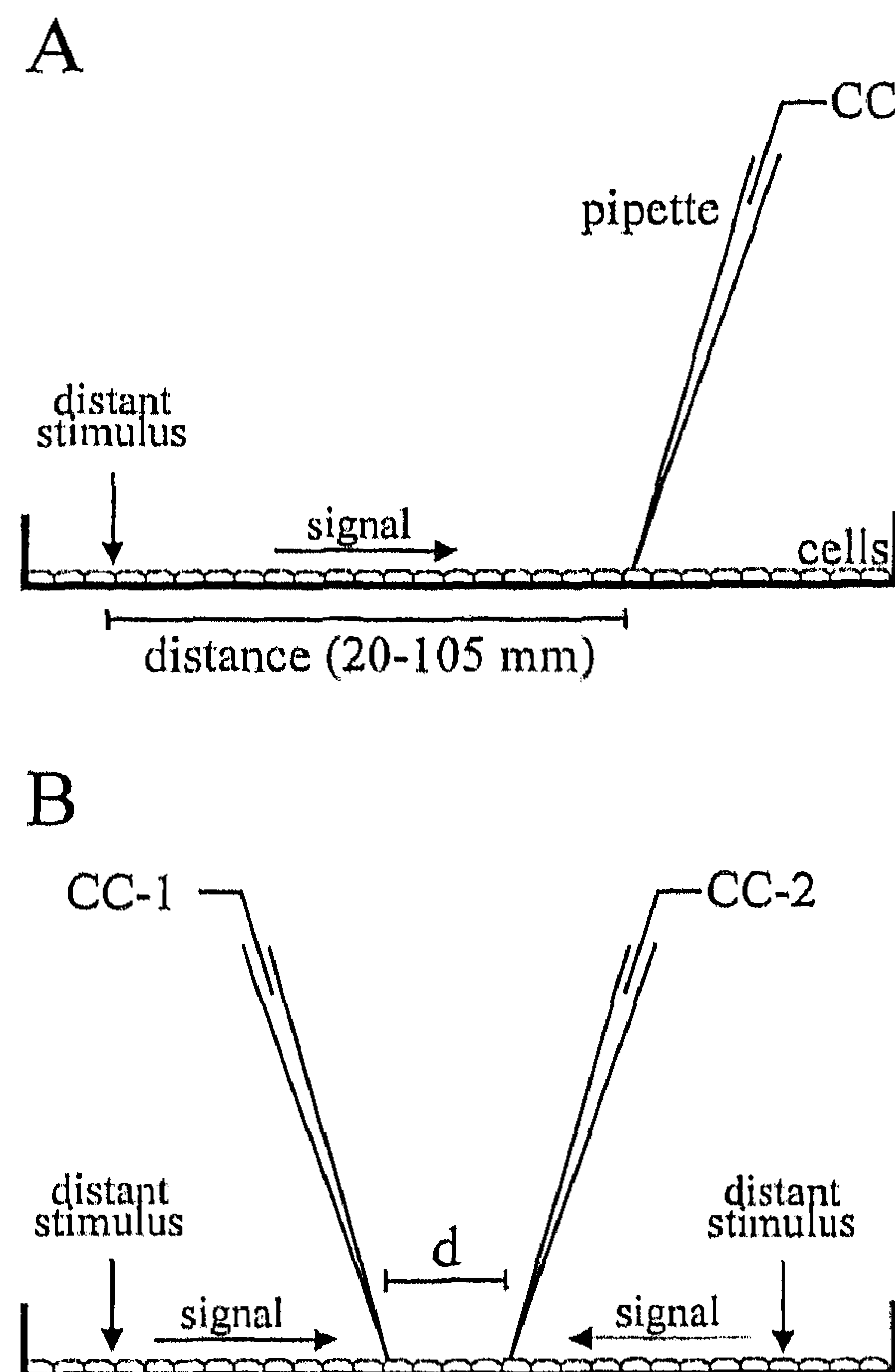


Fig. 1. Experimental setup. A: a cell in a monolayer was patched in whole cell configuration in current-clamp (CC) mode. A depolarizing stimulus was given at a certain distance (20–105 mm) from recording pipette, resulting in depolarization of only a small part of monolayer. Propagation of signal through monolayer results in a measurement of depolarization in patched cell. B: 2 cells from a monolayer at a certain distance ( $d$ ) were patched (CC-1 and CC-2). Propagation of a distantly applied stimulus results in a delay in signal between the 2 patched cells.

## RESULTS

We recently showed that spontaneous repetitive membrane potential depolarizations were observed in monolayer cultures of density-arrested NRK cells (9). In contrast to those density-arrested cells, spontaneous action potential-like depolarizations were never seen in quiescent NRK fibroblasts. However, perfusion of BK to monolayers of cells could induce a fast action potential-like depolarization (spike; Fig. 2A) that preceded the sustained depolarization evoked by BK. We have previously shown that this sustained depolarization, which

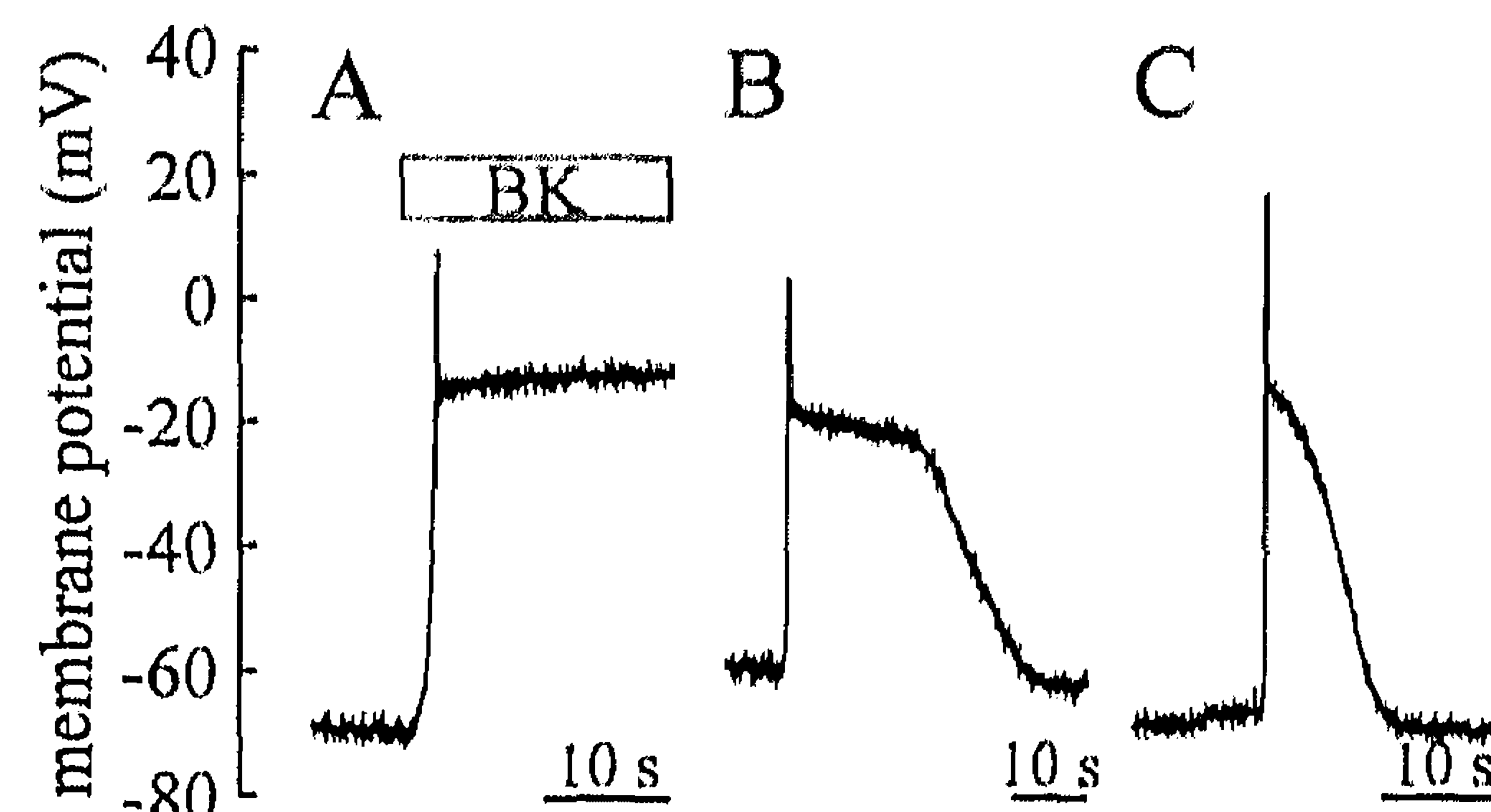


Fig. 2. Action potentials in monolayers of normal rat kidney (NRK) cells. A: spike depolarization preceding long-lasting depolarization evoked by perfusion of bradykinin (BK) in normal DF (1:1 DMEM-Ham's F-12) medium, which contains 1.8 mM  $\text{Ca}^{2+}$ . Perfusion causes a direct exposure of cells to 10 nM BK. B: action potential evoked by distant application (cf. Fig. 1) of 5  $\mu\text{l}$  of 10 nM BK that prevented direct exposure to BK in cells near measuring pipette (total bath volume, 2–20 ml). C: action potential evoked by distant exposure to 5  $\mu\text{l}$  of 124 mM  $\text{K}^+$  in medium containing 1.0  $\mu\text{M}$  BAY K 8644.

could last up to 15 min, is caused by an increase in a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance (8) due to the release of  $\text{Ca}^{2+}$  from intracellular stores (1).

The spike preceding the long-lasting depolarization by BK resembled an action potential, and, to investigate whether action potentials could be induced in quiescent NRK cells by BK, the effect of BK was measured distant from the site of its application in confluent quiescent monolayers of these cells. By the application of a small volume ( $5 \mu\text{l}$ ) of 10 nM BK in a large volume of medium (2.5–20 ml) it was ensured that only a small part of the monolayer was exposed to stimulatory concentrations of the agonist. In cells of the monolayer that were located at a distance far enough to prevent direct activation by a stimulatory concentration of BK (cf. Fig. 1A; distant stimulation), an action potential could be induced (Fig. 2B). This action potential could also be induced by depolarization of a small part of the monolayer by the distant addition of a small volume of an elevated extracellular  $\text{K}^+$  concentration ( $[\text{K}^+]_e$ ; 124 mM; Fig. 2C).

A possible effect of diffusion of BK to the site of measurement was ruled out by three facts. 1) When the small volume of 10 nM BK used for distant stimulation was first diluted to the final concentration after total diffusion in the bath, no depolarization was observed. Therefore, the dilution that occurs with distant stimulation causes substimulatory levels of BK (see also Fig. 4C). Concentrations of BK  $< 1$  nM have no effect on the membrane potential of NRK cells (de Roos, van Zoelen, and Theuvenet, unpublished observations). 2) In cases in which no action potential was evoked by distant addition of BK no distant depolarization was observed either. Also, when the propagating action potential was blocked with felodipine, no direct depolarization by BK was observed (cf. Fig. 3D). 3) A depolarization directly evoked by the distant application of a high concentration of BK ( $5 \mu\text{l}$  of 0.5 mM BK at 20 mm) resulting from a diffusion from the site of application to the site of measurement took  $> 1$  min to appear, whereas an action potential in this case had already been observed within 4 s. This shows that diffusion of BK to the site of measurement in a bath with no perfusion is much slower than the propagation velocity of the action potential (see also Fig. 4A). Therefore, it is concluded that the distant addition of a small volume of BK or  $\text{K}^+$  has no direct effect on the patched cell in which the action potential is measured.

These distantly evoked action potentials were seen in quiescent NRK cells in  $\sim 10\%$  of the monolayer cultures when tested with normal  $\text{Ca}^{2+}$ -containing media. The probability of the occurrence of the action potentials could be increased (to  $\sim 25\%$ ) by incubating the cells with the L-type  $\text{Ca}^{2+}$  channel activator (10) BAY K 8644 (Fig. 2C), indicating that L-type channels are involved in the generation of the action potential. However, the incidence of the action potentials could be markedly increased to almost 100% when media were used in which  $\text{Ca}^{2+}$  was replaced by  $\text{Sr}^{2+}$ . In this respect, quiescent NRK cells differ from density-arrested cells

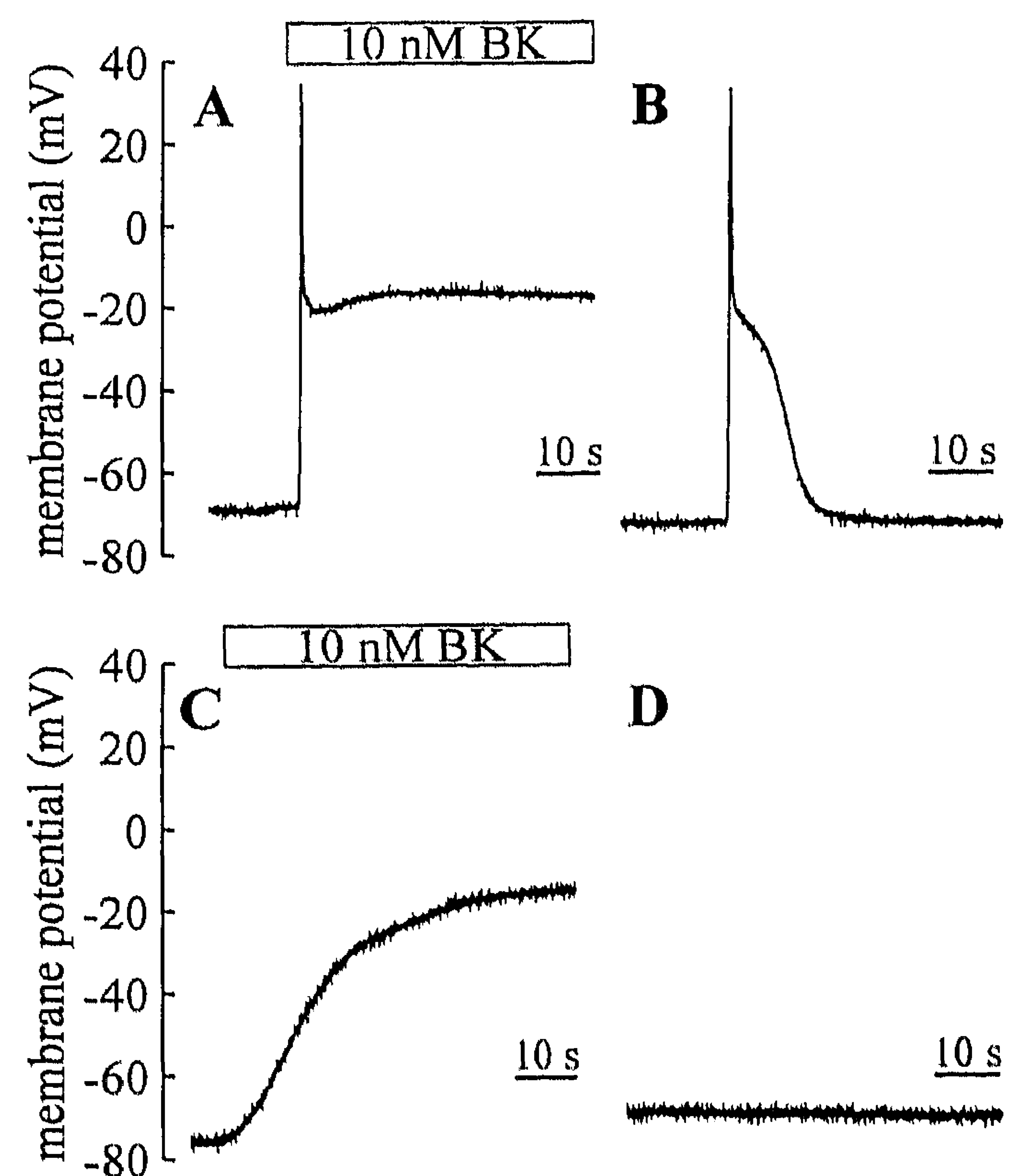


Fig. 3. Action potentials in  $\text{Sr}^{2+}$ -containing media. A: effect of perfusion of 10 nM BK on membrane potential in NRK fibroblasts, causing a direct exposure of cells to BK. B: effect of distant stimulation with  $5 \mu\text{l}$  BK in cells that were located at a distance (105 mm) from site of stimulation (cf. Fig. 1A). C and D: effect of direct (C) and distant (D) stimulation with BK in presence of  $1.0 \mu\text{M}$  felodipine. Nifedipine ( $1.0 \mu\text{M}$ ) had same effect as felodipine (not shown). All measurements were performed in media containing 3 mM  $\text{Sr}^{2+}$  with no  $\text{Ca}^{2+}$  added. Traces are typical for at least 6 experiments.

in which spontaneous action potentials occur in physiological media.

The ability of  $\text{Sr}^{2+}$  to substitute for  $\text{Ca}^{2+}$  indicated a role for extracellular  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels, since it is well known that  $\text{Sr}^{2+}$  can permeate  $\text{Ca}^{2+}$  channels (16). Because action potentials could be consistently generated in media in which  $\text{Sr}^{2+}$  substituted for  $\text{Ca}^{2+}$ , we decided to use  $\text{Sr}^{2+}$ -containing media to study the mechanism underlying the generation and propagation of the action potential. An additional benefit of  $\text{Sr}^{2+}$ -containing media is the better solubility of  $\text{Sr}^{2+}$  in bicarbonate-based buffers, thus allowing an increase in the divalent cation concentration in the medium.  $\text{Ba}^{2+}$  could not be used as a charge carrier, since  $\text{Ba}^{2+}$  blocks  $\text{K}^+$  channels (16) and thus depolarized NRK cells (not shown).

Figure 3A shows the effect of direct stimulation by BK on the membrane potential of quiescent NRK fibroblasts in  $\text{Sr}^{2+}$ -containing media. Also, in these media, perfusion of BK to monolayers induced a fast depolarizing spike (cf. Fig. 2A) followed by a sustained depolarization. The distant stimulation by BK induced an action potential (Fig. 3B) similar to the ones in  $\text{Ca}^{2+}$ -containing media, although the peak values of the action potential became more pronounced when the cells were incubated in media containing elevated concentrations of  $\text{Sr}^{2+}$ . This action potential was characterized by a fast upstroke followed by a plateau phase, after which membrane potential quickly returned to resting levels. The duration of the action potential (measured from onset to repolarization to  $-50$  mV) varied from monolayer to monolayer, with values be-

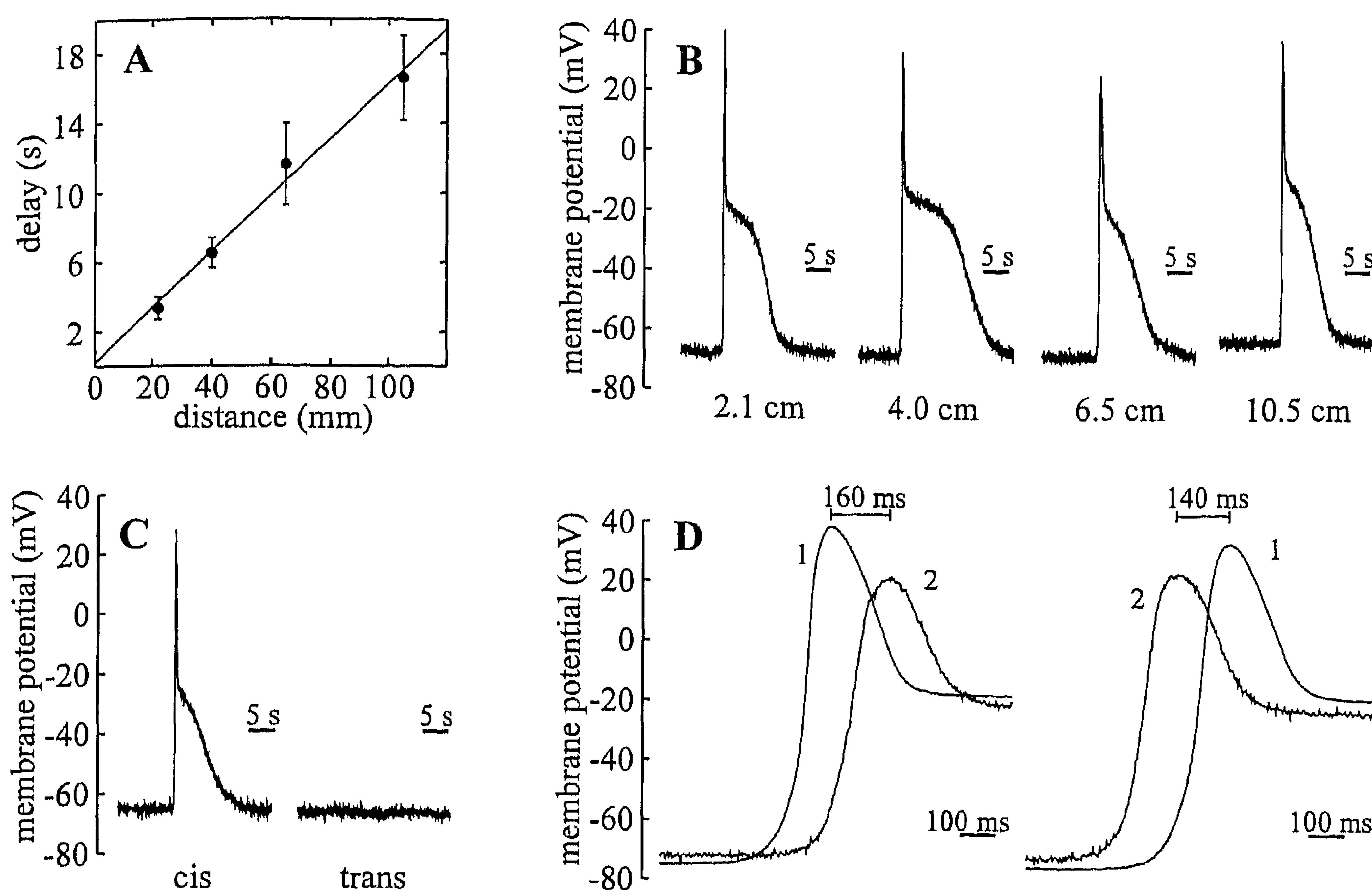


Fig. 4. Propagation of action potentials in monolayers of NRK cells. *A*: delay of action potential (means  $\pm$  SD;  $n = 8$ ) as a function of distance from patch pipette; 5  $\mu\text{l}$  of 124 mM  $\text{K}^+$  was added at indicated distances from recording pipette. Similarly, addition of 5  $\mu\text{l}$  of 10–100 nM BK evoked action potentials, but with a slightly larger delay. *B*: action potentials recorded after induction at different distances. *C*: action potential induction in a monolayer that was divided in half by a 3-cell-wide scratch; *cis*, recording pipette was on same side of scratched monolayer as place of induction; *trans*, action potential was induced on one side of monolayer and recording pipette was placed on other side. *D*: propagation of action potential. Two cells 700  $\mu\text{m}$  from each other were patched. A propagating action potential was evoked by distant application of high  $\text{K}^+$ , in line with 2 patched cells (cf. Fig. 1*B*), in such a way that action potential first reached patched cell 1 and then cell 2 (left) or vice versa (right). All measurements were done with 3 mM  $\text{Sr}^{2+}$  in medium.

tween 4 and 35 s and a mean ( $\pm$  SE) of  $16.2 \pm 8.3$  s ( $n = 34$ ). Once initiated, the action potential propagated in an all-or-none fashion, independently of the distantly applied stimulatory concentration of either  $\text{K}^+$  or BK, characteristic of an action potential (see also Fig. 4*B*).

The L-type  $\text{Ca}^{2+}$  channel blocker felodipine (27, 30) completely blocked the fast transient spike in cells located at the site of application of BK without affecting the sustained depolarization (Fig. 3*C*). The action potential was also blocked by the L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (27, 28) (not shown). These results indicate that opening of L-type  $\text{Ca}^{2+}$  channels is responsible for the spike depolarization but not for the sustained depolarization caused by BK. Moreover, felodipine and nifedipine completely blocked the action potential in cells that were not directly exposed to BK (Fig. 3*D*), showing that opening of L-type  $\text{Ca}^{2+}$  channels is necessary for the appearance of the action potential.

To determine the conduction velocity of the propagating action potential, action potentials were induced at several distances from the recording pipette (cf. Fig. 1*A*), and the resulting delay was measured. Figure 4*A* shows that this delay increased linearly with the distance and corresponds to a conduction velocity of 6.1 mm/s ( $r^2 = 0.994$ ). At all distances tested, the shape of the action potentials was similar, showing that the action potential propagated over very long ( $>105$  mm) distances in an all-or-none fashion (Fig. 4*B*). The monolayers that were used for the stimulation at 105 mm had a diameter of 14 cm and contained  $\sim 1 \times 10^6$  cells. Therefore, local depolarization can evoke an action potential that transiently depolarizes millions of cells. The requirement for intercellular communication for the propagation of the action potential was shown by the interruption of the monolayer by a three-cell-wide scratch (Fig. 4*C*). In this case, action potentials could only be measured when the recording pipette was

on the same (*cis*) side as the site of induction of the action potential. The propagation of the signal was also demonstrated by using two electrodes that were up to 900  $\mu\text{m}$  apart (cf. Fig. 1*B*). An action potential was induced by the distant application of  $\text{K}^+$  on either side of the two electrodes used, causing the propagating action potential first to reach the pipette nearest the site of depolarization. Figure 4*D* shows the resulting delays in the measurement of the action potential. The time delay measured in this setup corresponds to a conduction velocity of  $5.4 \pm 0.8$  mm/s (mean  $\pm$  SD;  $n = 5$ ), which is in good agreement with the value obtained from Fig. 4*A*. These results show that the action potential propagates at a constant speed through the monolayer.

The apparent involvement of L-type  $\text{Ca}^{2+}$  channels that emerges from the findings in Figs. 2 and 3 pointed to a direct role of divalent cation influx through  $\text{Ca}^{2+}$  channels in the generation of the action potential. Consequently, it may be expected that the peak value of the action potentials will be sensitive to changes in the external divalent cation concentration. The dependence of the peak potential on  $[\text{Sr}^{2+}]_e$  is shown in Fig. 5*A*. The peak potential increased linearly with the logarithm of  $[\text{Sr}^{2+}]_e$ . The slope of the graph is 29 mV/10-fold increase ( $r^2 = 0.996$ ) in the  $[\text{Sr}^{2+}]_e$ , as expected for a  $\text{Sr}^{2+}$  electrode (16). This indicates that the spike depolarization is solely caused by increased  $\text{Sr}^{2+}$ , and therefore  $\text{Ca}^{2+}$ , permeability.

L-type  $\text{Ca}^{2+}$  channels are activated by depolarizations beyond a threshold value of  $-10$  to  $-20$  mV (16, 28). Because well-coupled monolayers cannot be uniformly voltage clamped (2), increasing concentrations of  $\text{K}^+$  were perfused to the cells to progressively depolarize the cells. Figure 5*B* shows that only concentrations of  $\text{K}^+$  that depolarized the cells beyond  $-15$  mV were able to produce the all-or-none action potentials, which is similar to the reported threshold value of L-type  $\text{Ca}^{2+}$

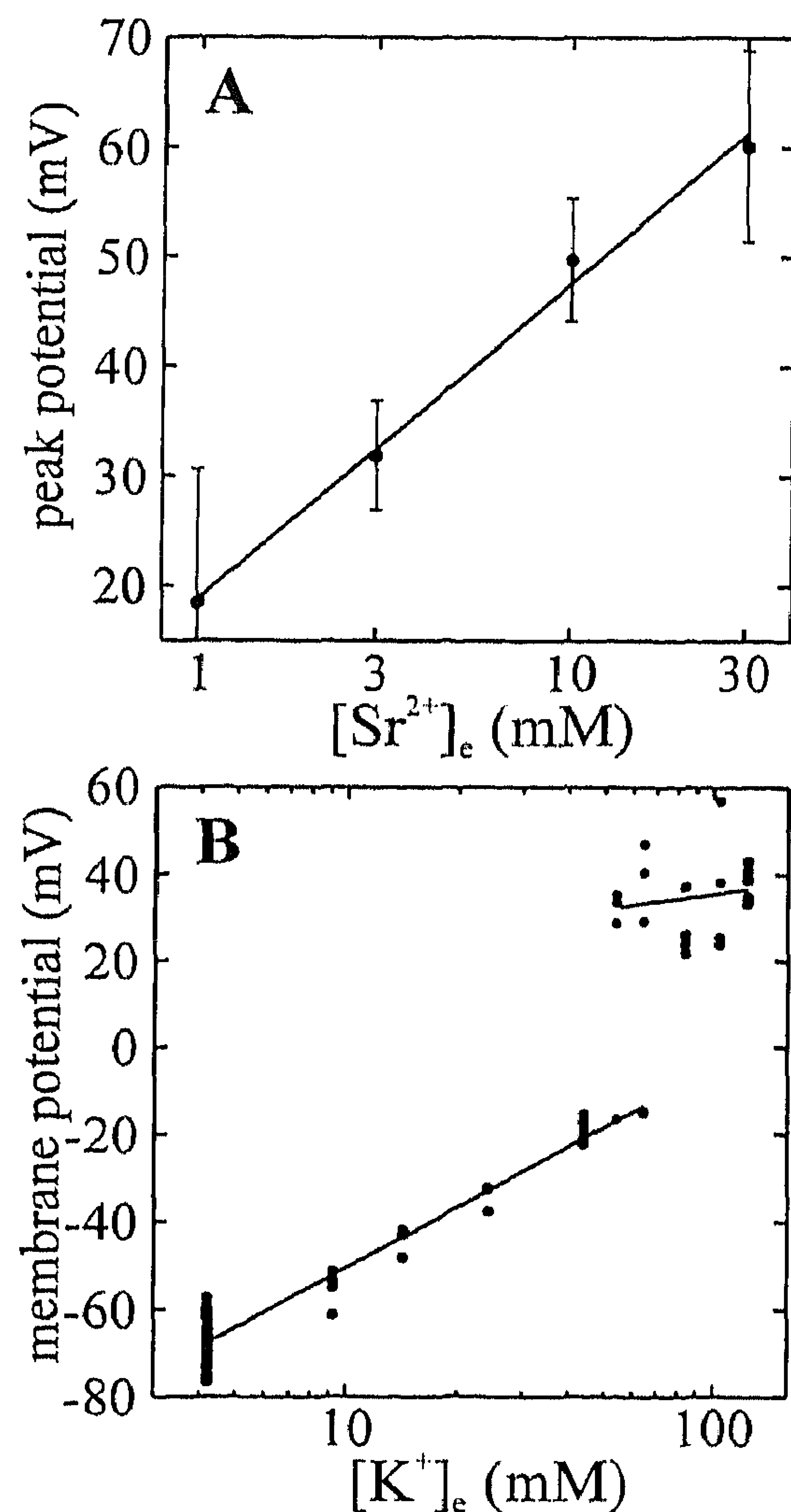


Fig. 5. Identification of  $\text{Sr}^{2+}$ -conducting channels. A: relation between peak value of action potential and extracellular  $\text{Sr}^{2+}$  concentration ( $[\text{Sr}^{2+}]_e$ ; means  $\pm$  SD;  $n = 6$ ). B: determination of threshold membrane potential for generation of action potential. Indicated  $[\text{K}^+]_e$  were rapidly perfused (3 ml/min) to bath. Data points show maximal depolarization evoked by  $[\text{K}^+]_e$ . Medium contained 3 mM  $\text{Sr}^{2+}$  with no added  $\text{Ca}^{2+}$ . When  $\text{SrCl}_2$  (A) or  $\text{KCl}$  (B) concentration was raised,  $\text{NaCl}$  concentration was adjusted to maintain osmolarity.

channels (16, 28). We recently showed that, in single trypsinized NRK cells, L-type voltage-dependent  $\text{Ca}^{2+}$  currents can be evoked with a threshold value of about  $-20$  mV and a peak current at 0 mV (9).

Low  $[\text{Na}^+]_e$  had no significant effect on the shape or duration of the distantly evoked action potential (Fig. 6B; duration of action potential,  $19.9 \pm 12.3$  s; peak,  $34.9 \pm 13.7$  mV;  $n = 5$ ), and neither did the potent  $\text{Na}^+$  channel blocker (16) tetrodotoxin (Fig. 6C; duration of action potential,  $14.2 \pm 10.7$  s; peak,  $30.8 \pm 4.8$  mV;  $n = 4$ ), which demonstrates that there is no  $\text{Na}^+$  component to the action potential. Low- $\text{Cl}^-$  media (Fig. 6D) changed the shape of the plateau phase of the action potential without affecting the duration ( $11.6 \pm 2.2$  s;  $n = 4$ ) and slightly affected the peak of the depolarizing spike peak ( $15.8 \pm 3.5$  mV;  $n = 4$ ), showing that the plateau phase is caused by an increase in  $\text{Cl}^-$  conductance of the plasma membrane. Buffering  $[\text{Ca}^{2+}]_i$  by loading the cells with the  $\text{Ca}^{2+}$  chelator BAPTA abolished the plateau phase (Fig. 6E; duration  $1.6 \pm 0.4$  s; peak  $25.4 \pm 7.5$  mV;  $n = 9$ ). These results indicate that the plateau phase involves a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance. The overall effect of a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance is therefore a lengthening of the  $\text{Ca}^{2+}$  action potential. In BAPTA-loaded cells, action potentials could still be induced by a high  $[\text{K}^+]_e$  but not by BK,

showing that the depolarization by BK is  $[\text{Ca}^{2+}]_i$  dependent. In summary, these results show that the upstroke of the action potential is solely mediated by a  $\text{Ca}^{2+}$  influx and is therefore a  $\text{Ca}^{2+}$  action potential (12), whereas the plateau phase is caused by an increased  $\text{Ca}^{2+}$ -mediated  $\text{Cl}^-$  conductance.

The involvement of L-type  $\text{Ca}^{2+}$  channels was further supported by the finding that action potentials could be induced by the distant stimulation with the L-type  $\text{Ca}^{2+}$  channel activator BAY K 8644 (10) (Fig. 6F). Thus, in  $\text{Ca}^{2+}$ -containing media, incubation with BAY K 8644 favors the induction of action potentials by a depolarizing stimulus (Fig. 3C), but in  $\text{Sr}^{2+}$ -containing media BAY K 8644 can evoke action potentials by itself, indicating an increased activity of L-type  $\text{Ca}^{2+}$  channels in  $\text{Sr}^{2+}$ -containing media.

The involvement of voltage-dependent  $\text{Ca}^{2+}$  channels in the action potential implied an influx of  $\text{Ca}^{2+}$  (or  $\text{Sr}^{2+}$  when this ion was used as a charge carrier) through these channels during an action potential and therefore an increase in  $[\text{Ca}^{2+}]_i$  or  $[\text{Sr}^{2+}]_i$ . Dynamic video imaging with the fluorescent probe fura 2 was used to simultaneously monitor the influx of  $\text{Sr}^{2+}$  or  $\text{Ca}^{2+}$  of  $\sim 100$  cells of the monolayer. Fura 2 can be used to measure not only  $\text{Ca}^{2+}$  influx but also  $\text{Sr}^{2+}$  influx, although the affinity of fura 2 for  $\text{Sr}^{2+}$  is lower than that for  $\text{Ca}^{2+}$  (20). Figure 7A shows the average increase in  $[\text{Ca}^{2+}]_i$  in all measured ( $\sim 100$ ) cells after the distant induction of an action potential in normal,  $\text{Ca}^{2+}$ -containing me-

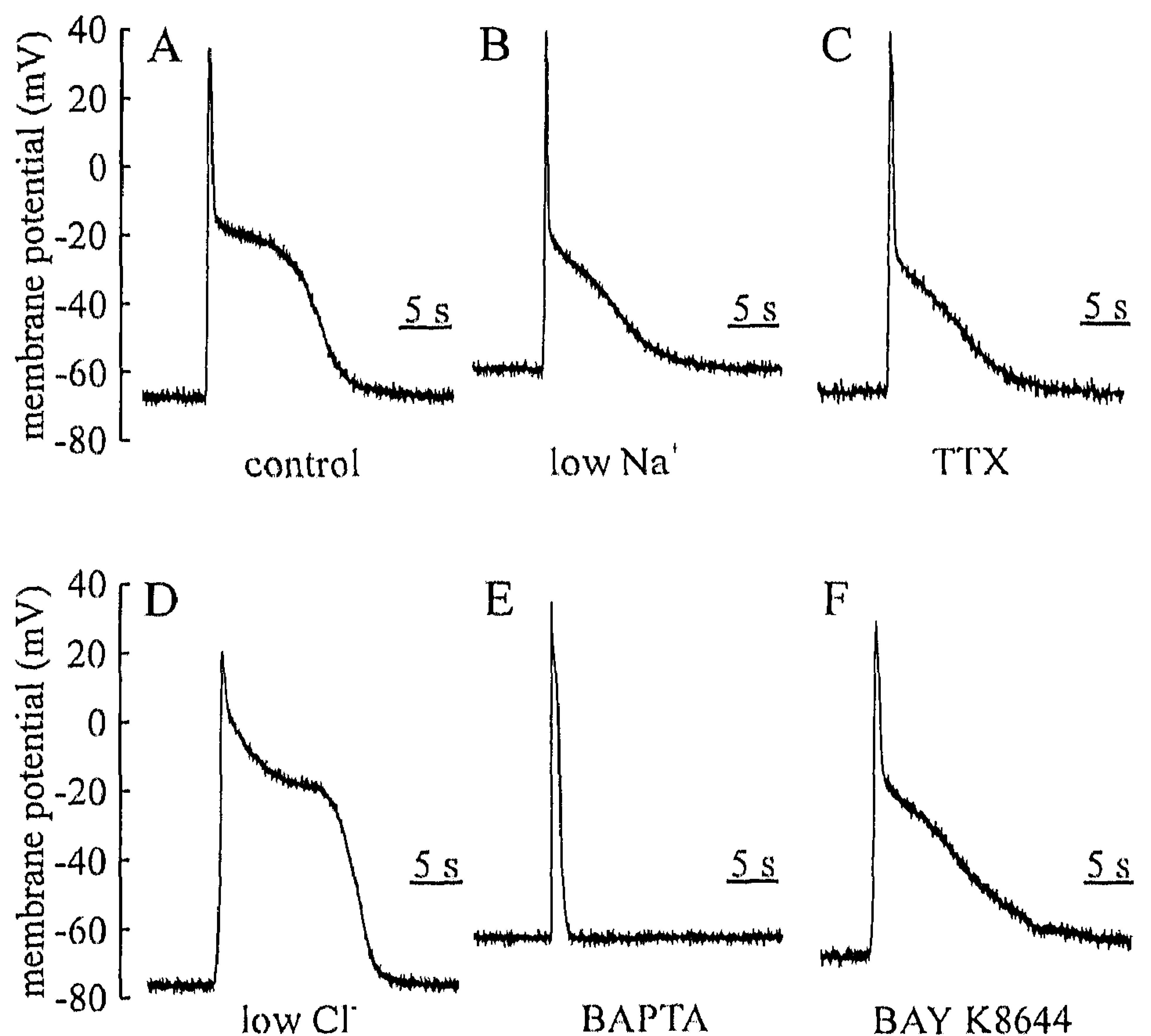


Fig. 6. Characterization of action potential. Action potentials were evoked by distant (20–105 mm) addition of  $10 \mu\text{l}$  of 124 mM  $\text{K}^+$ , unless otherwise noted (bath volume, 2.5 ml). Distant stimulation with  $10 \mu\text{l}$  of 10 nM BK had a similar effect. A: control. B: action potentials evoked in medium containing 9 mM  $\text{Na}^+$ . C: action potential in presence of  $1.0 \mu\text{M}$  tetrodotoxin (TTX). D: action potential evoked in 5 mM  $\text{Cl}^-$ . E: action potential in cells loaded with BAPTA before stimulation with  $\text{K}^+$ . BK was unable to evoke a  $\text{Ca}^{2+}$  transient or action potential in these BAPTA-loaded cells (not shown). F: Action potential evoked by  $1.0 \mu\text{M}$  BAY K 8644 (—) alone. All media contained 3 mM  $\text{Sr}^{2+}$  with no  $\text{Ca}^{2+}$  added.

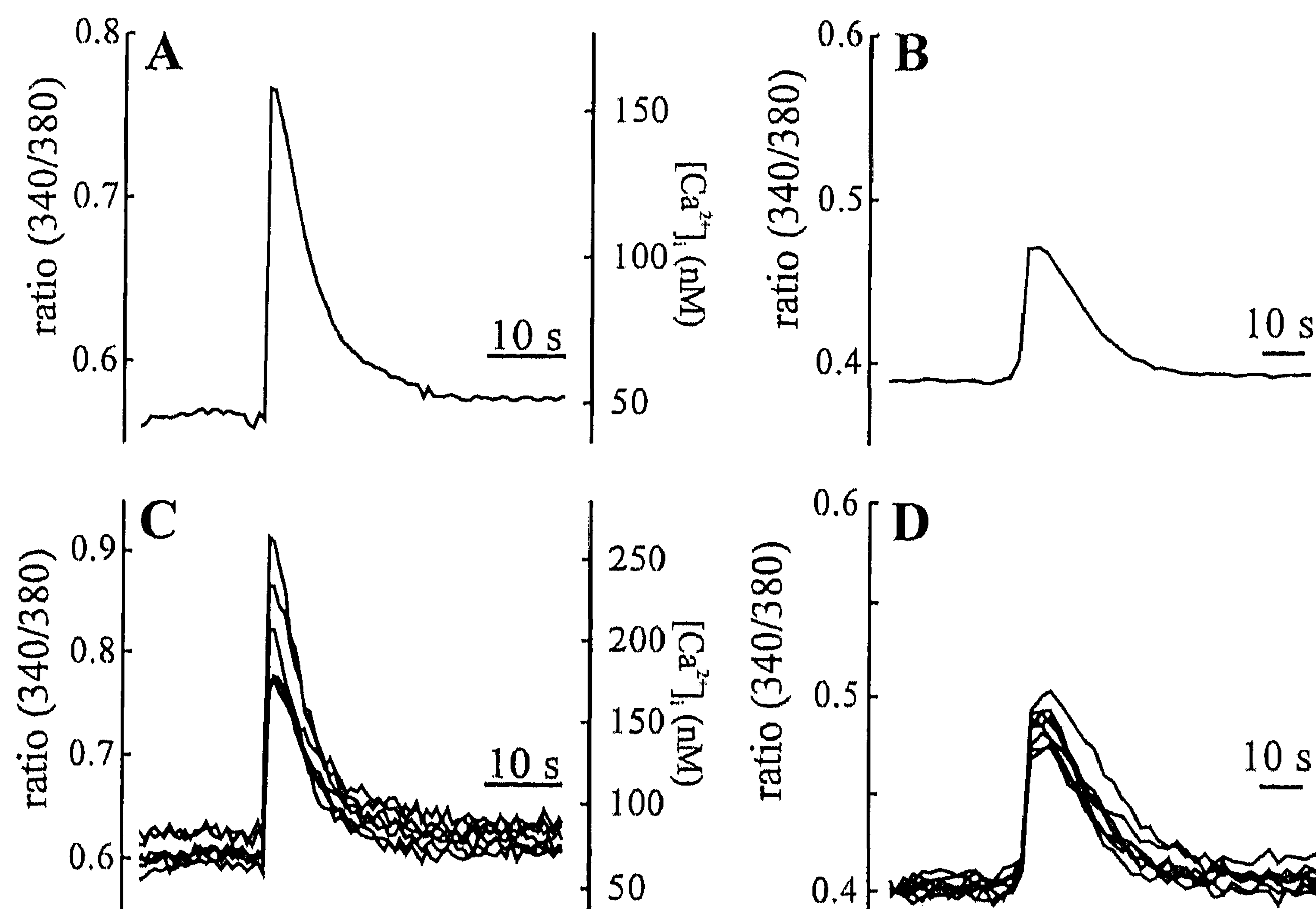


Fig. 7.  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$  influx measurements during an action potential. *A*: increase in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) after distant induction of an action potential in normal DF medium (containing 1.8 mM  $\text{Ca}^{2+}$ ) with 1.0  $\mu\text{M}$  BAY K 8644. Average increase in  $[\text{Ca}^{2+}]_i$  of  $\sim 100$  cells in monolayer ( $0.16 \text{ mm}^2$ ) after initiation of an action potential by local depolarization with 5  $\mu\text{l}$  124 mM  $\text{K}^+$  at 20 mm distance is shown. *B*: changes in fura 2 fluorescence by influx of  $\text{Sr}^{2+}$  through  $\text{Ca}^{2+}$  channels were used as a measure of  $\text{Ca}^{2+}$  influx (20). *C* and *D*: increase in  $[\text{Ca}^{2+}]_i$  and  $[\text{Sr}^{2+}]_i$ , respectively, in 8 individual cells that were randomly chosen from measured cells.

dium. During an action potential, the  $[\text{Ca}^{2+}]_i$  rose about threefold from a resting concentration of 50 nM. Figure 7C shows the  $[\text{Ca}^{2+}]_i$  response in eight individual cells, which were randomly chosen from all the measured cells. Figure 7C gives the  $\text{Ca}^{2+}$  response in cells with an average diameter of  $\sim 30 \mu\text{m}$  that were up to 300  $\mu\text{m}$  apart. These results demonstrate that the propagating action potential causes a virtually synchronous transient increase in  $[\text{Ca}^{2+}]_i$  by influx of  $\text{Ca}^{2+}$  in all cells of the monolayer. Also, action potentials could be evoked in media containing  $\text{Sr}^{2+}$  (cf. Fig. 3). Figure 7, B and D, shows the synchronous increases in  $[\text{Sr}^{2+}]_i$  caused by the propagating action potential in these  $\text{Sr}^{2+}$ -containing media. The transient increases in  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$  in Fig. 7 show that  $\text{Sr}^{2+}$  influx can be used to monitor  $\text{Ca}^{2+}$  influx. The delay between the induction and measurement of the  $\text{Ca}^{2+}$  transient or action potential (cf. Fig. 4A) was similar (2–3 s at distance of 20 mm). The durations of the increases in  $[\text{Ca}^{2+}]_i$  and  $[\text{Sr}^{2+}]_i$  were, respectively,  $12.3 \pm 3.7 \text{ s}$  ( $n = 7$ ) and  $25.8 \pm 9.9 \text{ s}$  ( $n = 7$ ), similar to the duration of changes in membrane potential during an action potential. These results show that depolarization of only a small part of the monolayer is sufficient to generate a propagating electrical signal in the monolayer that is transduced into a chemical signal, namely an almost synchronized transient increase in  $[\text{Ca}^{2+}]_i$  throughout the entire monolayer.

## DISCUSSION

The finding that monolayers of fibroblasts are excitable elucidates a new aspect of fundamental importance in the signaling of these cells, since these cells were hitherto considered to be a classic example of nonexcitable cells (4, 14, 23). The present data show that local application of physiological concentrations of an agonist like BK can induce a fast transient increase in  $[\text{Ca}^{2+}]_i$  in large numbers of cells that were not directly exposed to the stimulus. The  $\text{Ca}^{2+}$  action potentials reported here provide a mechanism for inter-

cellular communication that is several orders of magnitude faster than the reported  $\text{Ins}(1,4,5)\text{P}_3$ -dependent intercellular  $\text{Ca}^{2+}$  waves in fibroblasts and nonexcitable cells (20–50  $\mu\text{m}/\text{s}$ ; see Ref. 26), although slower than action potentials in nerve (1–100 m/s) or muscle (0.1–1 m/s), probably due to the electrical parameters of the fibroblast monolayer and the channels involved (16).

Electrical coupling between cells is a prerequisite for the intercellular propagation of action potentials. Fibroblast (or fibroblast-like) cells can be electrically coupled to each other (15, 18, 25) and may even form three-dimensional communicating networks in vivo (19). Fibroblasts can also be electrically coupled to other cells (17), and in this way networks of fibroblasts may play a central role in the synchronous or coordinated behavior of tissues. For example, fibroblasts are electrically coupled to myocytes and can transmit action potentials (24), but fibroblasts can also rescue excitability of dysgenic myotubes (6). We hypothesize that fibroblasts play an active role in the initiation and transduction of electrical signals involved in the coordination of multicellular activities.

In animal cells, action potentials depend on a regenerative depolarization caused by voltage-dependent ion channels that open on depolarization. Depolarization with either BK or high extracellular  $\text{K}^+$  evoked action potentials in NRK cells. Because the resting membrane potential is determined by a  $\text{K}^+$  conductance and is therefore dependent on the concentration gradient of  $\text{K}^+$ , elevating the extracellular  $\text{K}^+$  concentration directly depolarizes the cell. BK, however, releases  $\text{Ca}^{2+}$  from intracellular stores, leading to an increase in  $[\text{Ca}^{2+}]_i$ , which opens a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance with a concomitant depolarization (8). Therefore, by opening a  $\text{Cl}^-$  conductance via an increase in  $[\text{Ca}^{2+}]_i$ , BK indirectly depolarizes the cells. BK induces a propagating action potential but, in contrast to high  $\text{K}^+$ , does not induce an action potential in cells directly exposed to BK (de Roos et al., unpublished observa-

tions); transduction of the depolarization to neighboring cells seems to be the trigger for the propagating action potential. The action potential induced by perfusing BK to the bath (Figs. 2A and 3A) is a combination of distant stimulation (when BK enters the bath), which evokes an action potential, and direct stimulation (when BK reaches the cells), which causes the long-lasting depolarization.

The following mechanism for the generation of action potentials is proposed. Depolarization by extracellular  $\text{K}^+$  directly depolarizes the cells beyond the threshold value for L-type  $\text{Ca}^{2+}$  channels, causing these channels to open. In the case of BK, transduction to neighboring cells of a depolarization caused by the opening of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels activates L-type  $\text{Ca}^{2+}$  channels in these cells. Opening of  $\text{Ca}^{2+}$  channels generates an influx of  $\text{Ca}^{2+}$  in the cells, with a concomitant subsequent depolarization toward the equilibrium potential for  $\text{Ca}^{2+}$ . Further transduction of this depolarization to neighboring cells via gap junctions results in the regenerative opening of  $\text{Ca}^{2+}$  channels in these cells, resulting in active propagation of the signal through the whole monolayer. The influx of  $\text{Ca}^{2+}$  during the action potential opens a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  conductance responsible for the plateau phase. Because the  $[\text{Ca}^{2+}]_i$  seems to play a role in the maintenance of the plateau phase of the action potential, simultaneous measurements of  $[\text{Ca}^{2+}]_i$  and membrane potential are needed to determine a possible threshold function of the  $[\text{Ca}^{2+}]_i$ .

The following evidence for the involvement of L-type  $\text{Ca}^{2+}$  channels was obtained: 1) the action potentials could be blocked by the well-characterized L-type  $\text{Ca}^{2+}$  channel blockers felodipine and nifedipine (27, 28, 30); 2) action potentials could be induced by the L-type  $\text{Ca}^{2+}$  channel activator BAY K 8644 (10, 28); and 3) the observed threshold value for opening corresponds well with the reported value for L-type  $\text{Ca}^{2+}$  channels in the literature (16, 28). Moreover, we recently showed directly by means of voltage-clamp measurements in single trypsinized NRK cells that these fibroblasts indeed possess L-type  $\text{Ca}^{2+}$  channels (9).

L-type  $\text{Ca}^{2+}$  channels have also been shown to be present in mouse BALB/c 3T3 (21), human foreskin (3), mouse Swiss 3T3 (5), and in REF52 fibroblasts (14), whereas in Swiss 3T3 cells T-type  $\text{Ca}^{2+}$  channels have also been found (22). So far, a function for these voltage-dependent  $\text{Ca}^{2+}$  channels in fibroblasts has been unclear. Our results suggest that they may function in the generation and propagation of action potentials.

L-type  $\text{Ca}^{2+}$  channels are inhibited by high  $[\text{Ca}^{2+}]_i$  (11), and  $\text{Ca}^{2+}$  action potentials are more easily evoked under conditions of low  $[\text{Ca}^{2+}]_i$  (12, 16). When  $\text{Sr}^{2+}$  was used as the charge carrier, with no  $\text{Ca}^{2+}$  added to the medium, action potentials in quiescent NRK cells could be consistently generated by exposure to either BK or an elevated  $[\text{K}^+]_e$ . Therefore, these  $\text{Sr}^{2+}$ -containing media have been used in this study to investigate the induction and propagation of action potentials in NRK cells. It is hypothesized that incubation of the cells in

low- $\text{Ca}^{2+}$  media, which results in a decrease of the  $[\text{Ca}^{2+}]_i$ , could be responsible for the loss of inhibition of the L-type  $\text{Ca}^{2+}$  channels by  $[\text{Ca}^{2+}]_i$ , causing them to open more easily. A block of the action potentials by high  $[\text{Ca}^{2+}]_i$  would be in agreement with the observation that the addition of a small volume of  $\text{K}^+$  directly to cells evokes an action potential in these cells, whereas depolarization by the local addition of a small volume of BK, which first causes an increase in the  $[\text{Ca}^{2+}]_i$ , does not evoke an action potential in the cells directly exposed to BK (de Roos et al., unpublished observations) but does evoke a propagating action potential in the remainder of cells that were not directly exposed to BK (this article).

The present findings show that activation of L-type  $\text{Ca}^{2+}$  channels can evoke action potentials in NRK fibroblasts. The action potentials reported here are similar to the spontaneous action potential-like depolarizations (9) seen in NRK cells that are grown to density arrest in the presence of EGF (1, 29), and it is concluded that  $\text{Ca}^{2+}$  action potentials underlie these spontaneous depolarizations. The cells used in this study to induce action potentials were made quiescent by the deprivation of serum, whereas density-arrested cells stop growing when they reach a critical cell density, similar to the growth inhibition of cells in vivo (29). Thus the ability of NRK fibroblasts to behave like an excitable tissue may depend on the appropriate environmental cues. The fact that density-arrested NRK monolayers exhibit spontaneous action potentials in normal  $\text{Ca}^{2+}$ -containing media may imply an increased activity of L-type  $\text{Ca}^{2+}$  channels at higher cell densities, and thus that activity of these  $\text{Ca}^{2+}$  channels is modulated by cell density. A role of the activity of  $\text{Ca}^{2+}$  channels in the incidence of action potentials is in agreement with the observation that, in  $\text{Ca}^{2+}$ -containing media, occurrence of action potentials in quiescent cells was markedly increased when they were incubated with the L-type channel activator BAY K 8644.

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