J. Membrane Biol. 124, 113–126 (1991)
002226319100137S

The Journal of
Membrane Biology
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Variety of Ca^{2+}-Permeable Channels in Human Carcinoma A431 Cells

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Summary. Patch-clamp methods were used to search for and characterize channels that mediate calcium influx through the plasma membrane of human carcinoma A431 cells. Here we present four Ca^{2+}-permeable channel types referred to as SG, G, I and BI. With 105 mM Ca^{2+} as the charge carrier, at 30–33°C their mean unitary conductances (in pS) are: 1.3 (SG), 2.4 (G), 3.7 (I) and 12.8 (BI). SG and G channels are activated by nonhydrolyzable analogues of guanosine 5-triphosphate (GTP) applied to the inside of the membrane, suggesting an involvement of G-proteins in the control of their activity. I and BI channels are activated by inositol 1,4,5-trisphosphate ($InsP_3$). G, I, BI and possibly SG channels are activated from the extracellular side of the membrane by epidermal growth factor (EGF) and histamine. It is assumed that all identified Ca^{2+} channels take part in the generation of the agonist-induced intracellular Ca^{2+} signal. The variety of Ca-channel types seems to be necessary to tune cell responses according to the respective type and level of an external signal, on the one hand, and to the functional state of the cell, on the other.

Key Words Patch clamp · carcinoma cell · receptor-operated, calcium-permeable channels · guanine nucleotide · inositol trisphosphate

Introduction

Calcium ions have been widely recognized as intracellular regulators (Rasmussen & Barrett, 1984). The increase of cytosol concentration of free calcium (Ca_i^{2+}) is an important mechanism of signal transduction in many cell types. This increase can arise either due to Ca^{2+} release from intracellular stores and/or to Ca^{2+} influx through the plasma membrane (Putney, 1987; Berridge & Irvine, 1989; Hallam & Rink, 1989). The principal pathways for Ca^{2+} influx seem to be calcium-permeable ionic channels. In nerve, muscle and other electrically excitable cells, Ca influx is mediated by voltage-gated Ca^{2+} channels (Reuter, 1986). In nonexcitable cells, mechanisms of Ca^{2+} entry are less certain. Studies of Ca-ion fluxes (Johns et al., 1987; Nishimoto et al., 1987; Zschauer et al., 1987), Ca^{2+}-dependent membrane functions (Morris et al., 1987; MacDougall, Grinstein & Gelfard, 1988; Mozhayeva, Naumov & Kuryshev, 1989*a*,*b*; Pandiella et al., 1989; Avdonin, Cheglakov & Tkachuk, 1990) and measurements of Ca_i^{2+} with Ca^{2+}-sensitive dyes (Shapiro, Adams & Niederhuber, 1985; Kato et al., 1987; Volpi & Berlin, 1988; Merrit, Jacob & Hallam, 1989) have suggested that plasma membrane of many cell types contains Ca^{2+}-permeable channels different from long-known voltage-gated ones. In view of the importance of Ca^{2+} influx as one of the mechanisms of cellular signaling, it is necessary to identify possible ways of Ca^{2+} entry and to characterize their functional properties and their mechanisms of control. Several types of receptor-operated calcium channels have been found in recent patch-clamp studies (Kuno et al., 1986; von Tscharner et al., 1986; Matsunaga et al., 1988; Kuno, Okada & Shibata, 1989; Mozhayeva, Naumov & Kuryshev, 1989*c*).

In this paper, a single-channel description of an ensemble of Ca^{2+}-permeable channels in A431 human carcinoma cells is given. These cells have been widely used in studies concerned with different aspects of transmembrane signaling. In particular, the studies of the calcium signaling (Macara, 1986; Moolenaar et al., 1986; Hepler et al., 1987; Gonzales et al., 1988) suggest that the membrane of these cells bears calcium-permeable channels responsive to EGF, histamine, bradykinin and some other agonists.

Materials and Methods

Cells

Experiments were performed on cultured human epidermoid carcinoma A431 cells. The cell culture was obtained from the Cell Culture Collection (Institute of Cytology, USSR). The culture was maintained in glass flasks in basal Eagle's medium supple-

mented with 10% bovine or fetal calf serum and 30 μg/ml gentamycin. For experiments the cells were plated on coverslips (0.5 × 0.5 cm) and grown in the same medium in 6% CO_2 humidified atmosphere to half or near confluency. One to four hr before the experiment the cells were deprived of serum.

Solutions

The pipettes were filled with 100 mM $CaCl_2$ (or $SrCl_2$ or $BaCl_2$) plus 5 mM $Ca(OH)_2$/HEPES mixture (pH 7.4). Two bath solutions were used in the experiments: sodium solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 Tris-HCl (pH 7.4) and potassium solution different from the first one in that it contained 145 mM KCl instead of the NaCl/KCl mixture. EGF or histamine was added either to the potassium bath or to the pipette solutions. The cytosol-like control solutions contained (in mM): 145 KCl (K aspartate, glutamate, arobonate), 10 HEPES/KOH (pH 7.3), 5 EGTA/KOH, 1.5 $CaCl_2$ (Ca^{2+} = $<10^{-7.5}$), and 1 $MgCl_2$. When necessary, the concentration of free Ca^{2+} was adjusted to higher values either with EGTA (Ca_i^{2+} = $<10^{-6}$ M) or N-hydroxyethyl-ethylene-diamine triacetic acid (HEEDTA) ($10^{-6} < Ca_i^{2+} < 10^{-5}$ M) calcium buffers. Ca^{2+} concentrations were calculated using the stability constants taken from Bers and MacLeod (1988). Small portions of internal solutions with GTPγS (5′-O-(3-thiotriphosphate), GppNHp (5′-guanylyl-imidodiphosphate) and $InsP_3$ (inositol 1,4,5-trisphosphate) were kept frozen and thawed just before the experiment. GppNHp and histamine were from Serva; GTPγS, HEPES, and HEEDTA were from Sigma; EGTA was from Fluka; and $InsP_3$ was from Calbiochem. EGF from mouse submaxilliary glands was a gift of Dr. A.D. Sorkin (Sorkin, Teslenko & Nikolsky, 1988).

Patch-Clamp Method

Ionic currents were studied in cell-attached or inside-out configurations (Hamill et al., 1981). Micropipettes made of Pyrex glass were covered with Sylgard®, fire polished and had resistance of 8–12 MΩ. Coverslips with cells were fixed on the glass bottom of the working chamber (volume of about 60 μl) with the clip made of platinum-iridium wire. At the beginning of the experiment, the working chamber (bath) was filled with the external sodium solution; then, after the giga-seal had been formed, the sodium solution was substituted with the potassium solution to zero-cell resting potential. When working in inside-out configuration, the pipette tip with excised membrane was transferred to the tube-shaped microchamber (volume about 20 μl).

Experiments were carried out either at 20–23 or at 30–33°C.

The bath was grounded and the potential inside the pipette was clamped at the desired value. Membrane potential (V) is expressed with respect to the pipette potential value.

Current signal was recorded and stored using an analog tape recorder at bandwidth 1.2 or 2.5 kHz. The tape was replayed, and the signal was filtered with a 6-pole Bessel filter at corner frequencies from 1.5 to 0.1 kHz. The filter settings were chosen depending on unitary-current amplitudes, rate of open-closed transitions and background patch noise. The signal was then digitized at 1 to 10 kHz and inserted into a computer.

The next step in signal processing was calculation of amplitude histograms for successive time intervals of 1 to 5 sec in duration. The amplitude histograms were used to determine unitary-current amplitudes (i) and probability for the channel to be open (P_o). The histograms consisted of two or more equally spaced peaks (*see* Fig. 1*b*), corresponding to zero, one, two, etc., channels open simultaneously. These peaks can be fitted by Gaussian curves, with the difference between means of neighboring peaks giving unitary-current amplitude. Alternatively, i values were determined from records of long, well-resolved events reproduced either on the screen of a digital oscilloscope or on paper by an X-Y pen recorder.

P_o was calculated using the formula: $P_o = \langle I \rangle / (i \cdot N_f)$, where $\langle I \rangle$ is the mean current through the channels for a given time interval, i is the unitary-current amplitude, and N_f is the number of functional channels in the patch. $\langle I \rangle$ was calculated from amplitude histograms as the time integral of patch current above baseline. The number of functional channels (N_f) in the patch was assessed by the maximum number of simultaneous openings over the whole period of observation.

To estimate stimulating effects of channel activators, we compared maximum values of open probabilities for each condition (control, presence of agonists, intracellular messengers, etc.) over a certain time interval. Usually, the duration of this interval was 20 sec, hence ($P_{o(20)}$). Such a duration, on one hand, was long enough to smooth down the stochastic nature of channel activity and, on the other hand, allowed comparison of the levels of channel activity whether transient or prolonged.

When possible, distributions of open and closed times were calculated with single-channel events detected with the use of the half-amplitude threshold criterium (Colquhoun & Sigworth, 1983).

Averaged data are given as the mean ± SE (number of experiments).

Results

One of the characteristic features of this study was that channels of interest occurred very rarely in patches and, when present, were, as a rule, active for only a short time. Therefore, many patches had to be tested in order to obtain sufficient data to identify and characterize each channel type.

SG Channels

Figure 1 shows one representative experiment on an excised patch. The pulse-like inwardly directed current events indicate channel openings. Current amplitudes are very small, but, nevertheless, can be clearly distinguished from a basal level by proper filtering. Figure 1*b* shows a current-amplitude histogram at −50 mV, the most positive potential used in these experiments. The histogram consists of two clearly separated peaks corresponding to basal level (no open channel) and one open channel. The slope of the current-voltage relation corresponds to channel conductances as small as 1.3 pS. Inward currents under the conditions used could be carried either by anions going outwardly or Ca^{2+} (the only cation in the pipette) going inwardly. These possibilities would have been easily distinguished, had reversal potential of the currents been determined. However,

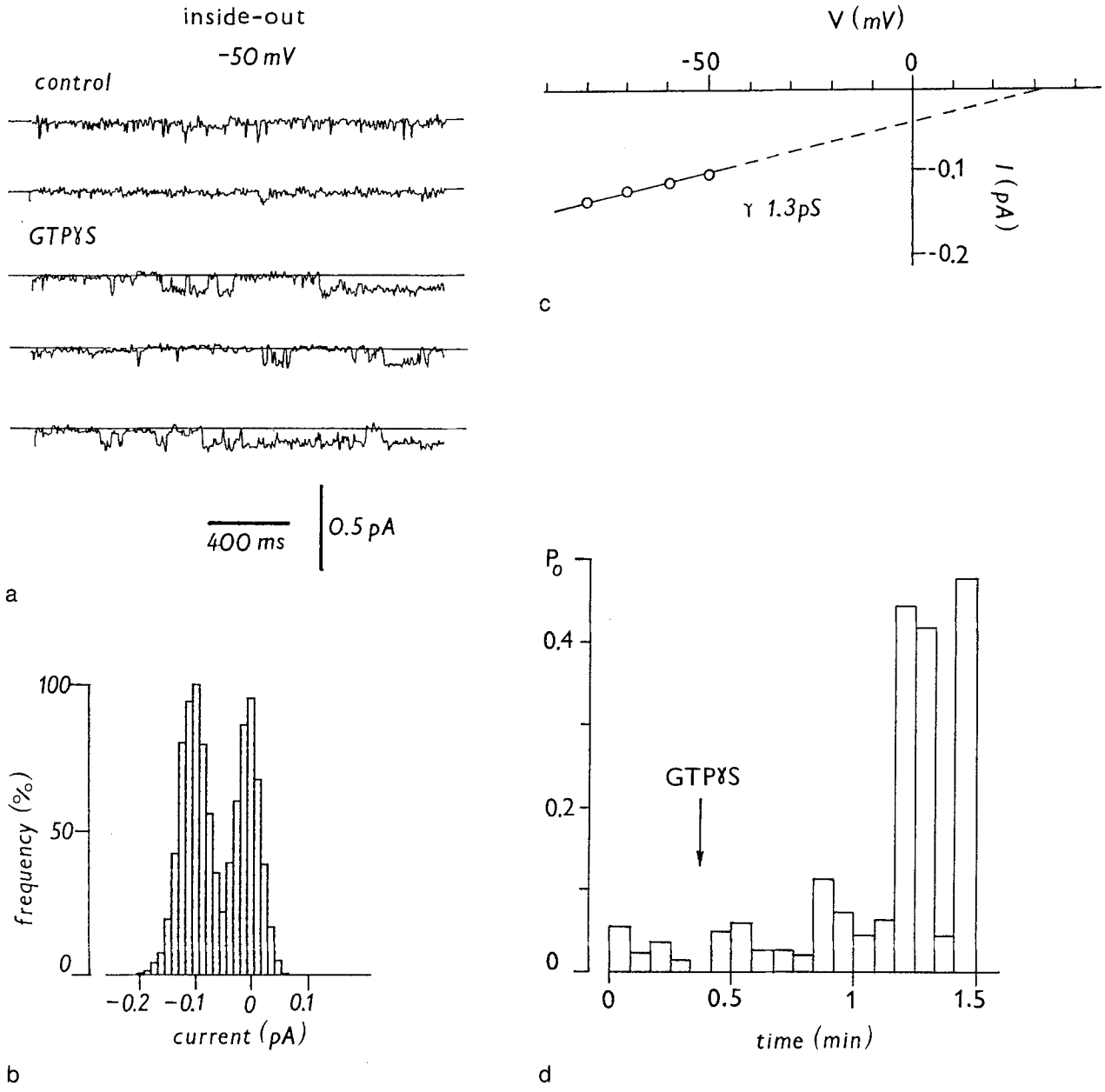


Fig. 1. Activation of SG channels with GTPγS in excised patch. (*a*) Current records at −50 mV before and after the addition of 100 μM GTPγS to the internal solution. Before reproducing on the X-Y plotter the current signal was filtered at 0.1 kHz. (*b*) Amplitude histogram constructed for 3-sec stretch of the record taken 50 sec after GTPγS application. The histogram can be fitted by a sum of two Gaussians (*not shown*) with the following parameters: 0.00 ± 0.020 pA (basal line) and 0.098 ± 0.025 pA (one channel open). Membrane potential was −50 mV; filter was 0.1 kHz. (*c*) Current-voltage relation for the same experiment. (*d*) Time course of the channel activity expressed in open probability before (shown only last 20-sec stretch) and after the GTPγS application (indicated by arrow). Glutamate was the internal solution. EGF (6.3 nM) was added to the pipette solution. Temperature 32°C.

with very small current amplitudes at potentials positive to −50 mV, we could estimate only an extrapolated reversal potential. It was positive, which argues against anionic selectivity of the channel; with 200 mM Cl^- in the pipette, the Cl^- equilibrium potential is expected to be negative, even if the intracellular Cl^- concentration was equal to that in the extracellular solution. Moreover, if the channel was anion selective, currents through it should depend on anion species in the internal solution. Currents shown in Fig. 1 were measured with K-glutamate as the major internal salt; the same current amplitudes

were obtained with KCl. It seems to be highly improbable that an anionic channel passes indiscriminately such sterically different anions as Cl^- and glutamate$^-$. Thus, it would be safe to assume that the channel under consideration is Ca^{2+} permeable.

It should be noted that the membrane of A431 cell contains low-conductive anionic (Cl^-) channels which will be described elsewhere. They could be easily distinguished from Ca^{2+}-permeable channels by the following features: their reversal potentials were always negative both in cell-attached and inside-out experiments, and they shifted toward more negative potentials upon the substitution of Cl^- with organic anions in artificial internal solution. Anionic channels were potential and Ca^{2+} dependent, with the activity increasing at more positive potentials and at larger Ca_i^{2+}.

Channels with a slope conductance between 1.2 to 1.4 pS, with a positive value of extrapolated reversal potential and insensitivity to anion species in artificial internal solutions were observed in nine experiments in which cell-attached and inside-out measurements were performed. In the latter case chloride, glutamate or aspartate as internal anions were used. We assume that these channels belong to the same type of Ca^{2+}-permeable channels, which we shall refer to as SG channels (SG stands for small guanine nucleotide sensitive). The fact that we describe here the results obtained in nine experiments does not mean that SG channels were not present in other patches. Judging by currents with amplitudes within the range typical for these channels, SG channels seemed to be present in 22 additional patches. However, the channels could not be identified rigorously because their conductances were not determined.

Channel activity was measured either at room temperature (seven experiments) or at 31–32°C (two experiments). Nevertheless, there was no significant difference in conductance values, which could be ascribed to the temperature, so we pooled together all data when calculating the mean value of 1.28 ± 0.02 pS (n = 9).

It can be seen from Fig. 1 that the channel openings were infrequent and short with control intracellular solution and were greatly augmented after the addition of a nonhydrolyzable analogue of GTP, GTPγS. The channel activity measured in $P_{o(20)}$ values (*see* Materials and Methods) increased 10 times in this experiment. In other inside-out experiments on patches containing SG channels, GTPγS or GppNHp (100 μM) increased $P_{o(20)}$ from 0.02 ± 0.01 to 0.38 ± 0.07 (n = 6). The intracellular messenger $InsP_3$ (5 μM) had no effect on SG-channel activity (three experiments).

The effect of GTP analogues on channel activity suggests the possibility that the SG channels are controlled by some receptor via a guanine nucleotide-binding protein (G-protein) (Gilman, 1987). Hence, SG channels would be expected to be responsive to Ca-mobilizing agonists such as EGF, histamine, etc. Eight of nine experiments presented here were performed with EGF added to the pipette solution. In two of them, $P_{o(20)}$ in the cell-attached configuration was rather high, exceeding 0.2 (*see* Fig. 2). EGF could have activated these channels in analogous experiments, but the activity was short lived, and therefore, could have been missed (for a more detailed discussion of such a possibility, see the section dealing with I channels). Additional experiments are necessary to clarify this point.

G Channels

Figure 3 shows one of the experiments favorable enough to demonstrate some important properties of this channel type both in cell-attached and then in inside-out configurations. In this experiment, EGF was added to the pipette solution, and apparently, due to this the channel activity in cell-attached configuration was notably higher than that in experiments without any agonist (*see below*). After the patch had been excised, the channel activity decayed and increased again after the application of GTPγS to the internal solution. The unitary current-voltage relation shows a conductance of 2.5 pS and, when extrapolated, intercepts the x-axis at about +30 mV. Current amplitudes were the same both in cell-attached and inside-out configurations. In the latter case, they remained unchanged upon substitution of glutamate with chloride in the internal solution. Thus, channels shown in Fig. 3 are, like SG channels, also Ca^{2+}-permeable. Ca^{2+}-permeable channels with similar conductances were activated by analogues of GTP in 24 experiments. In one experiment the same was seen with GTP. GTP analogues increased average $P_{o(20)}$ values from 0.019 ± 0.004 to 0.19 ± 0.03 (n = 24). At 21–23°C these channels had conductances from 1.9 to 2.6 pS (mean 2.27 ± 0.08 pS, n = 7) and at 30–33°C from 2.0 to 2.9 pS (mean 2.39 ± 0.07 pS, n = 16). We shall refer to them as G channels (G stands for guanine nucleotide). Ca^{2+}-permeable channels with conductances within these limits were observed in 49 additional cell-attached and inside-out experiments. In these experiments the responsiveness of the channels to GTP analogues were not examined; nevertheless, we assume that all of them belong to the G type, as well.

Without an agonist in the pipette the G channels were observed in 17 cell-attached patches. Their

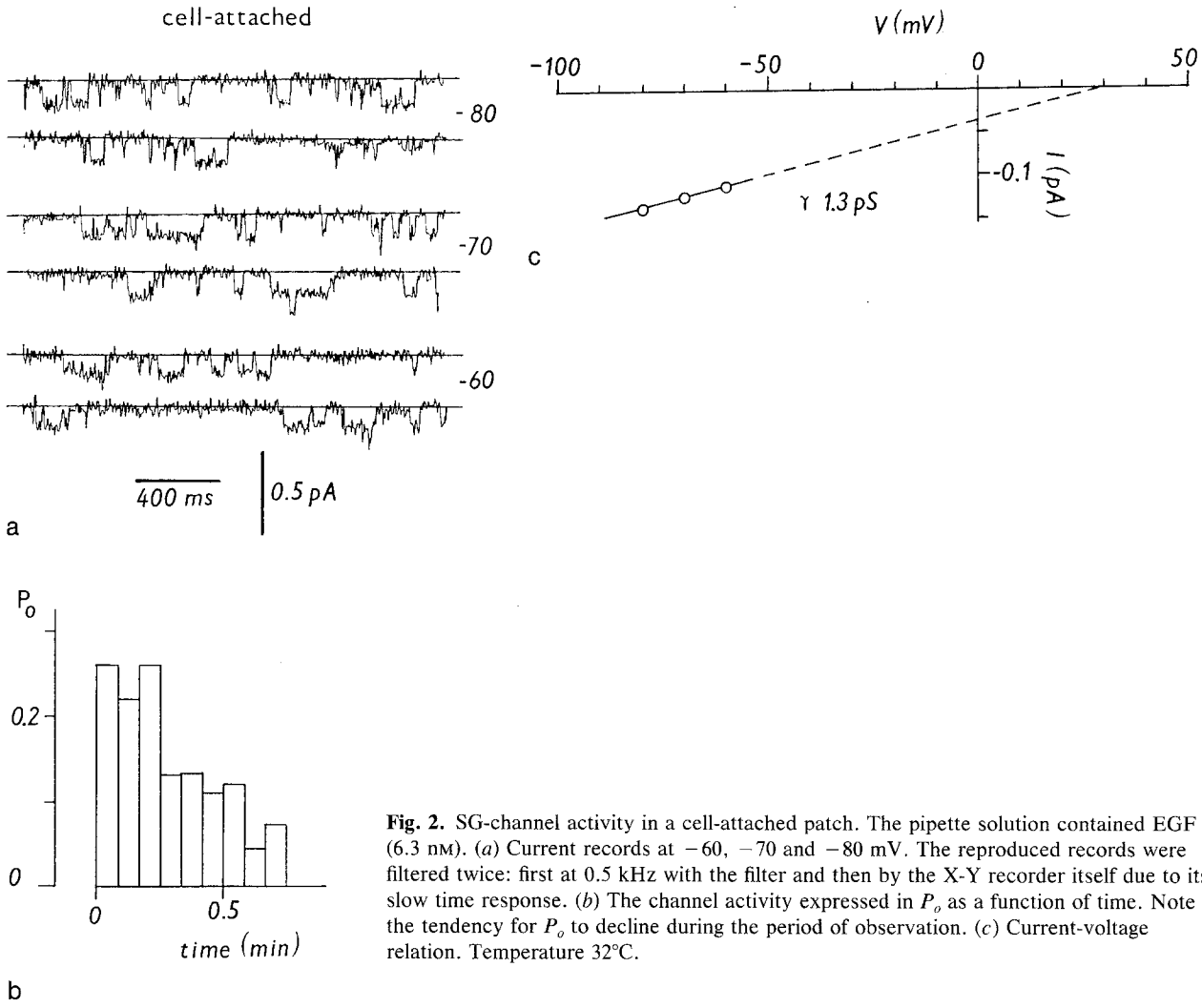


Fig. 2. SG-channel activity in a cell-attached patch. The pipette solution contained EGF (6.3 nM). (*a*) Current records at −60, −70 and −80 mV. The reproduced records were filtered twice: first at 0.5 kHz with the filter and then by the X-Y recorder itself due to its slow time response. (*b*) The channel activity expressed in P_o as a function of time. Note the tendency for P_o to decline during the period of observation. (*c*) Current-voltage relation. Temperature 32°C.

average $P_{o(20)}$ was calculated to be 0.018 ± 0.005. It should be noted that with low channel activity, as was typical for unstimulated patches, we could underestimate the number of channels in the patch, and thus, overestimate P_o (*see* Materials and Methods). Hence, the above $P_{o(20)}$ value should be taken as an upper limit for the basal channel activity. With EGF (3.7 to 8.3 nM) in the pipette the G channels were observed in 16 patches, $P_{o(20)}$ ranging from 0.02 to 0.83 (mean 0.17 ± 0.05), i.e., at least, nine times higher than without EGF. The G channels could be activated by histamine as well. Addition of histamine (25–100 μM) to the pipette resulted in a sixfold increase in G-channel activity (n = 5).

Open-time histograms were calculated in four experiments in which, on the one hand, channel activity was large enough and, on the other, there were no superpositions of channel openings. Each of the four distributions consisted of, at least, two parts. The first included most of the events and could be fitted by exponentials with time constants 4.9, 4.6, 6.2 and 9.0 msec. The second part consisted of more prolonged (tens and even hundreds of msec) events; it could not be quantitatively characterized because of an insufficient number of openings. Mean open times (T_o) for these experiments were calculated to be between 9.1 and 12.6 msec.

I Channels

Figure 4 presents an experiment on a patch which contained two channel types: G type and another, which we shall refer to as I type (I stands for inositol). Application of GTPγS resulted in activation of G channels (2.0 pS), which subsided within 35 sec (later, within 10 min of observation, there were four more bursts of activity of these channels not shown in the figure). Subsequent addition of 5 μM $InsP_3$ evoked a dramatic increase in the activity of channels with a conductance of 3.7 pS (prior to the addition of $InsP_3$ they opened very rarely). The activa-

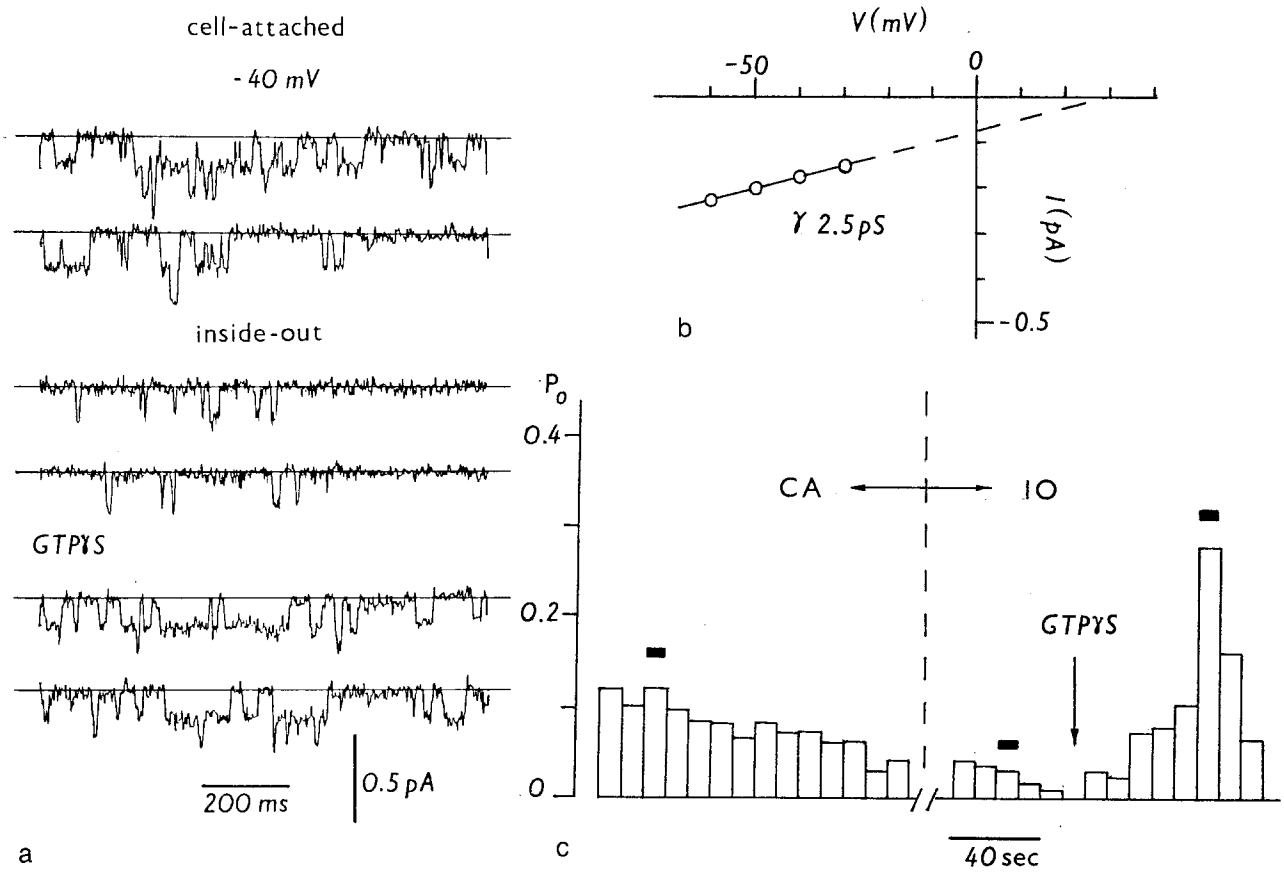


Fig. 3. Activity of G channels in cell-attached and inside-out configurations. (*a*) Representative current records first in cell-attached and then in excised patch configurations before and after the addition of GTPγS. (*b*) Current-voltage relation. Shown are only currents measured in the inside-out configuration with glutamate internal solution; the same current amplitudes were attained with chloride as the major anion (*not shown*). (*c*) The channel activity, expressed in P_o, in time. Filled rectangles indicate the time when the current records shown in *a* were taken. Note a decrease in the channel activity after the excision of the patch and its resumption after the GTPγS addition. Temperature 33°C.

tion of I channels with $InsP_3$ was observed in nine experiments when GTP analogues were applied prior to $InsP_3$. GTP analogues alone failed to produce a stimulating effect on I channels although they apparently modulated their activity. In five experiments $InsP_3$ alone evoked a weak, short-lived burst of I-channel activity, and in three of them a subsequent addition of GTP analogues caused an additional, more prolonged rise in channel activity.

Two metabolites of $InsP_3$, inositol 1,3,4,5-tetrakisphosphate (5 μM) and inositol 1,3,4 trisphosphate (2 μM), were tried in 139 inside-out experiments. These agents did not evoke or augment the channel activity in any of these experiments.

IP_3-sensitive channels (inside-out experiments) had conductances from 3.1 to 3.7 pS (mean 3.31 ± 0.12 pS, $n = 5$) at 20–23°C and from 3.4 to 4.0 pS (mean 3.70 ± 0.07 pS, $n = 7$) at 30–33°C. Channels with conductances within these limits were observed in 33 additional cell-attached and inside-out experiments. Though in these experiments the channels were not tested for the sensitivity to $InsP_3$, we assume that they belong to the I type.

As I channels are activated with $InsP_3$ applied to the intracellular side of the excised patch, they are suggested to be activated extracellularly by agonists inducing the $InsP_3$ release. Experiments in cell-attached configuration are in agreement with this suggestion. In the absence of an agonist in the pipette, I channels opened rarely: mean $P_{o(20)}$ was 0.02 ± 0.01 ($n = 10$). With EGF (3.7 to 8.3 nM), $P_{o(20)}$ ranged from 0.01 to 0.38, being on average 0.14 ± 0.04 ($n = 14$). The effect of EGF on I channels is illustrated by Fig. 5. The channel activity was high at the start of the observation period and then declined to nearly zero level. Such a time course suggests that in analogous experiments, the channel activity could have decreased significantly by the time measurements began because with the flow of pipette solution EGF reached the cell membrane a

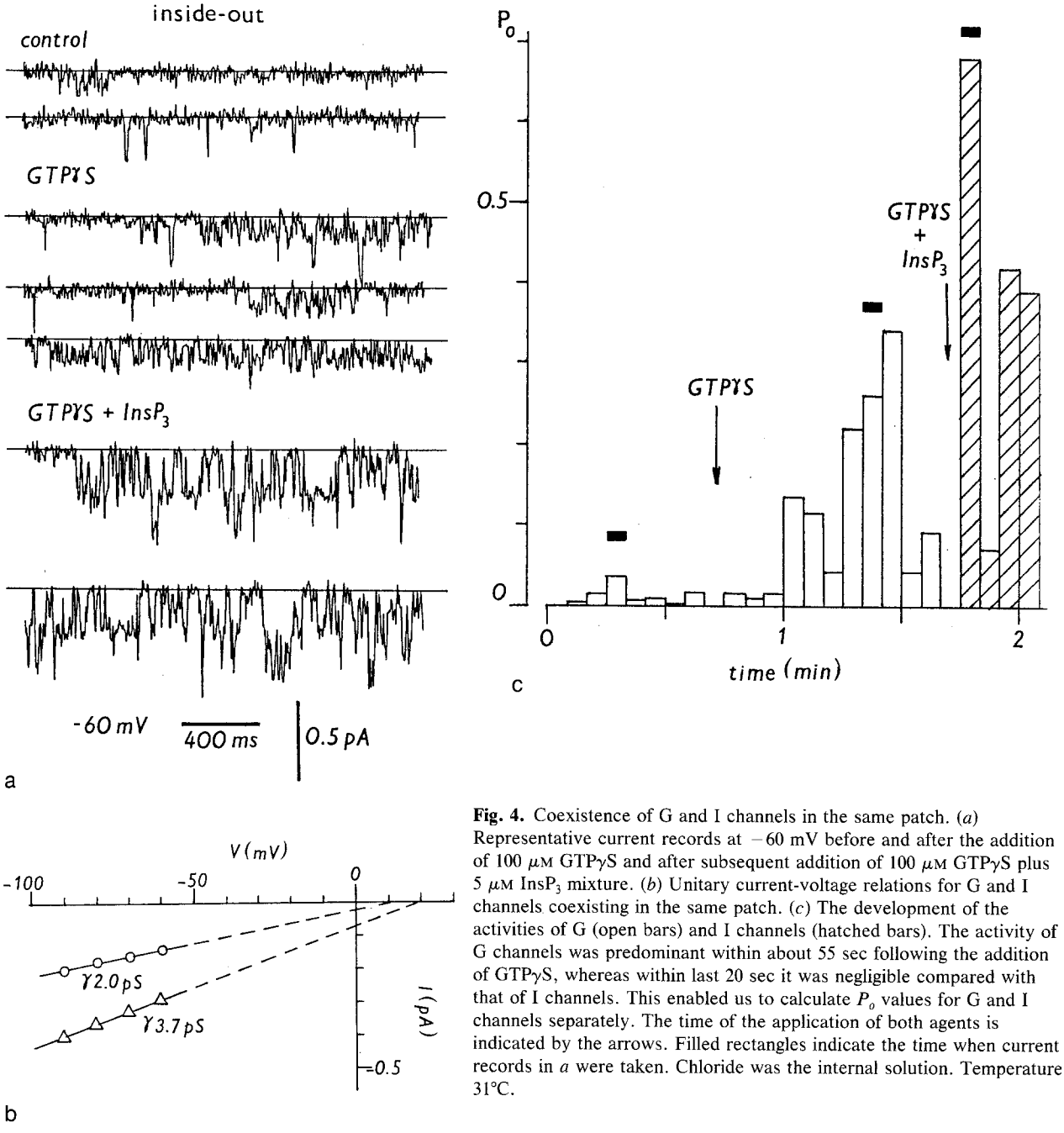


Fig. 4. Coexistence of G and I channels in the same patch. (*a*) Representative current records at −60 mV before and after the addition of 100 μM GTPγS and after subsequent addition of 100 μM GTPγS plus 5 μM $InsP_3$ mixture. (*b*) Unitary current-voltage relations for G and I channels coexisting in the same patch. (*c*) The development of the activities of G (open bars) and I channels (hatched bars). The activity of G channels was predominant within about 55 sec following the addition of GTPγS, whereas within last 20 sec it was negligible compared with that of I channels. This enabled us to calculate P_o values for G and I channels separately. The time of the application of both agents is indicated by the arrows. Filled rectangles indicate the time when current records in *a* were taken. Chloride was the internal solution. Temperature 31°C.

few seconds earlier than the pipette touched the cell and formed a giga-seal. For this reason the $P_{o(20)}$ value given above seems to underestimate the stimulating effect of EGF on I channels. The data are consistent with the notion that EGF does induce $InsP_3$ release from the patch membrane, and thereby, activates I channels, although this activation is short lived due to a limited store of the phosphatydylinositol 4,5-bisphosphate in it and $InsP_3$ diffusion away from the patch.

High level of I-channel activity was observed in five experiments with histamine (25–100 μM) and in one with bradykinin (1 μM) in the pipette.

The mean open times for I channels were calculated in five experiments. Their values were 6.0, 8.3, 16.8, 17.4 and 18.1 msec.

BI Channels

Figure 6 shows an experiment on an excised patch containing channels which pass inward currents of relatively large amplitudes even at small potentials.

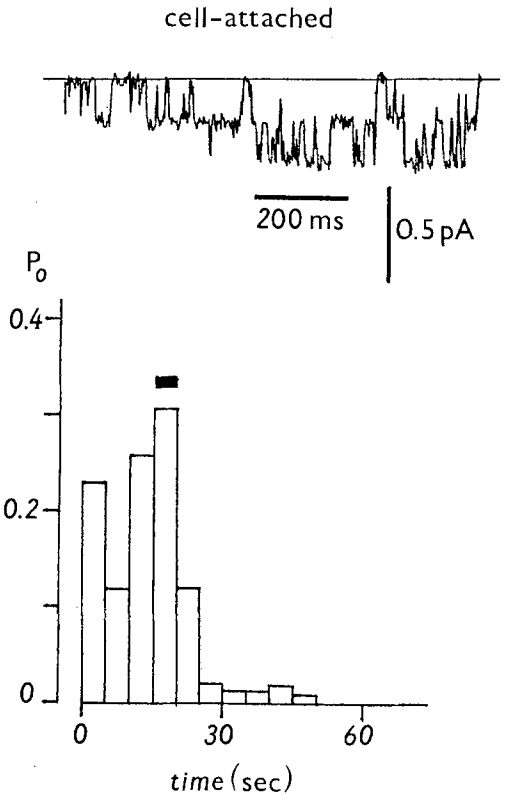


Fig. 5. Activation of I channels by addition of EGF to the pipette solution. Cell-attached configuration is shown. Inset shows a representative current record. Note that the channel activity nearly disappeared within 50 sec. Conductance of the channels in this patch was 3.6 pS; membrane potential was −50 mV. Temperature 33°C.

Thus, current measurements directly indicate that a reversal potential for these currents is positive. Moreover, current amplitudes were the same whether Cl^- or glutamate$^-$ was the major anion in the internal solution. Taken together these facts strongly suggest that the currents are carried by Ca^{2+} ions present in the pipette solution. The current-voltage relation has a slope conductance of about 10 pS at potentials between 0 and −30 mV. Channels with conductances from 9 to 14 pS were observed in 28 experiments; they seem to constitute a homogenous group. Judging from the insensitivity of currents to anion species and positive values of the respective reversal potentials they are Ca^{2+}-permeable channels. We shall refer to them as BI channels (I stands for $InsP_3$ sensitive and B stands for big to distinguish them from I channels). Their mean conductances were 10.3 ± 0.3 pS (n = 8) at 21–23°C and 12.8 ± 0.4 pS (n = 13) at 30–33°C.

In four experiments with 100 mM Sr^{2+} instead of Ca^{2+} in the pipette we observed channels with a mean conductance at room temperature of 13.5 ± 0.9 pS. They seem to belong to the BI type.

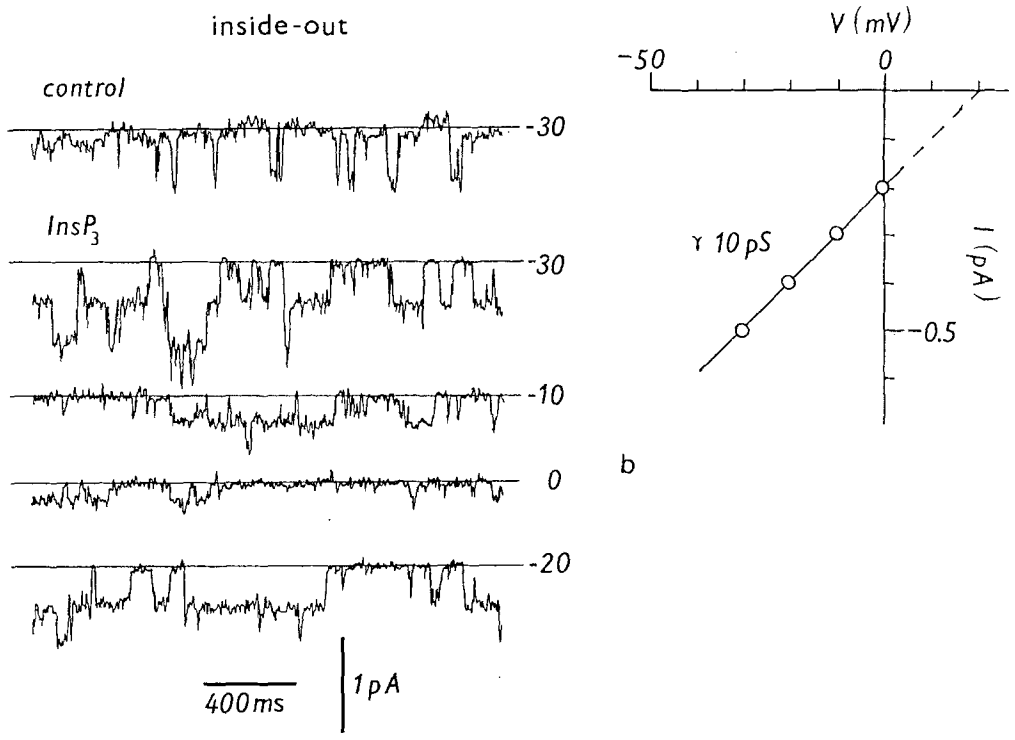
BI channels proved to be sensitive to the intracellular messenger $InsP_3$. It can be seen from Fig. 6 that the addition of 5 μM $InsP_3$ to the internal solution caused considerable increase in channel activity. $P_{o(20)}$ increased from about 0.07 to 0.37 in this experiment. Similar effects were evident in nine experiments; in five of them $P_{o(20)}$ was calculated to be 0.02 ± 0.01 and 0.22 ± 0.04 before and after the addition of 5 μM IP_3, respectively.

Figure 6 demonstrates another property of BI channels: voltage sensitivity. It can be seen that a shift of potential from −30 to −10 mV and then to 0 mV resulted in a decrease in the channel activity, and subsequent repolarization to −20 mV restored it. Similar phenomena were observed in two more experiments sufficiently long lasting to make it possible to compare the channel activity at different potentials.

Figure 7 shows one of the experiments in which EGF was added to the pipette solution. It can be seen that in a cell-attached patch the channel activity was rather high at the start of the observation period and then declined to virtually zero level. After the excision of the patch the activity was resumed by the application of $InsP_3$. BI-channel activity declining in time was observed in four more cell-attached experiments with EGF in the pipette. Such a transient time course of the BI-channel activity seems to be due to the EGF-induced production of $InsP_3$ and its subsequent diffusion from the patch membrane (*see* the section above).

In seven cell-attached experiments EGF (16 nM) was added to the bath solution. One such experiment is presented in Fig. 8. Before the EGF addition there were very rare current events within a 3-min period of observation; approximately 10 sec after the EGF addition into the bath inward currents became more frequent. A similar, though less pronounced, increase in BI-channel activity was observed in six additional experiments with EGF and in one experiment with histamine (100 μM).

Figure 9 shows open- and closed-time histograms obtained in an experiment with a patch containing one functional channel. The major part of open-time distribution could be reasonably described by a single exponential with the time constant equal to 1.0 msec. The remaining "tail" seemed to arise from unresolved short closings. Close-time distribution consisted of, at least, two clearly distinct parts: a fast and a slow one. The fast part can be fitted by an exponential with the time constant (τ_{cf}) equal to 0.7 msec. This component corresponds to short closings within bursts. The



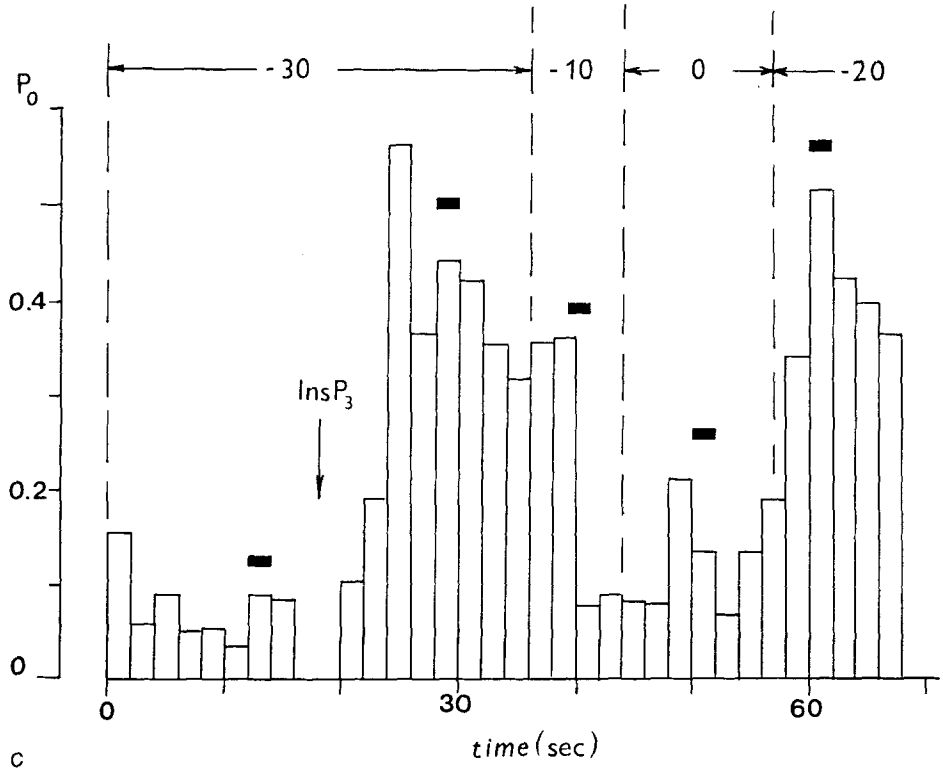


Fig. 6. BI-channel activity in inside-out patch. (*a*) Current records before and after the addition of 5 μM $InsP_3$ to the internal solution. Numbers on the right of the records indicate membrane potentials in mV. (*b*) Unitary current-voltage relation. (*c*) The channel activity expressed in P_o before and after $InsP_3$ application at −30, −10, 0 and −20 mV. Filled rectangles indicate the time when current records shown in *a* were taken. Glutamate was the internal solution. EGF (6.3 nM) was added to the pipette solution. Temperature 32°C.

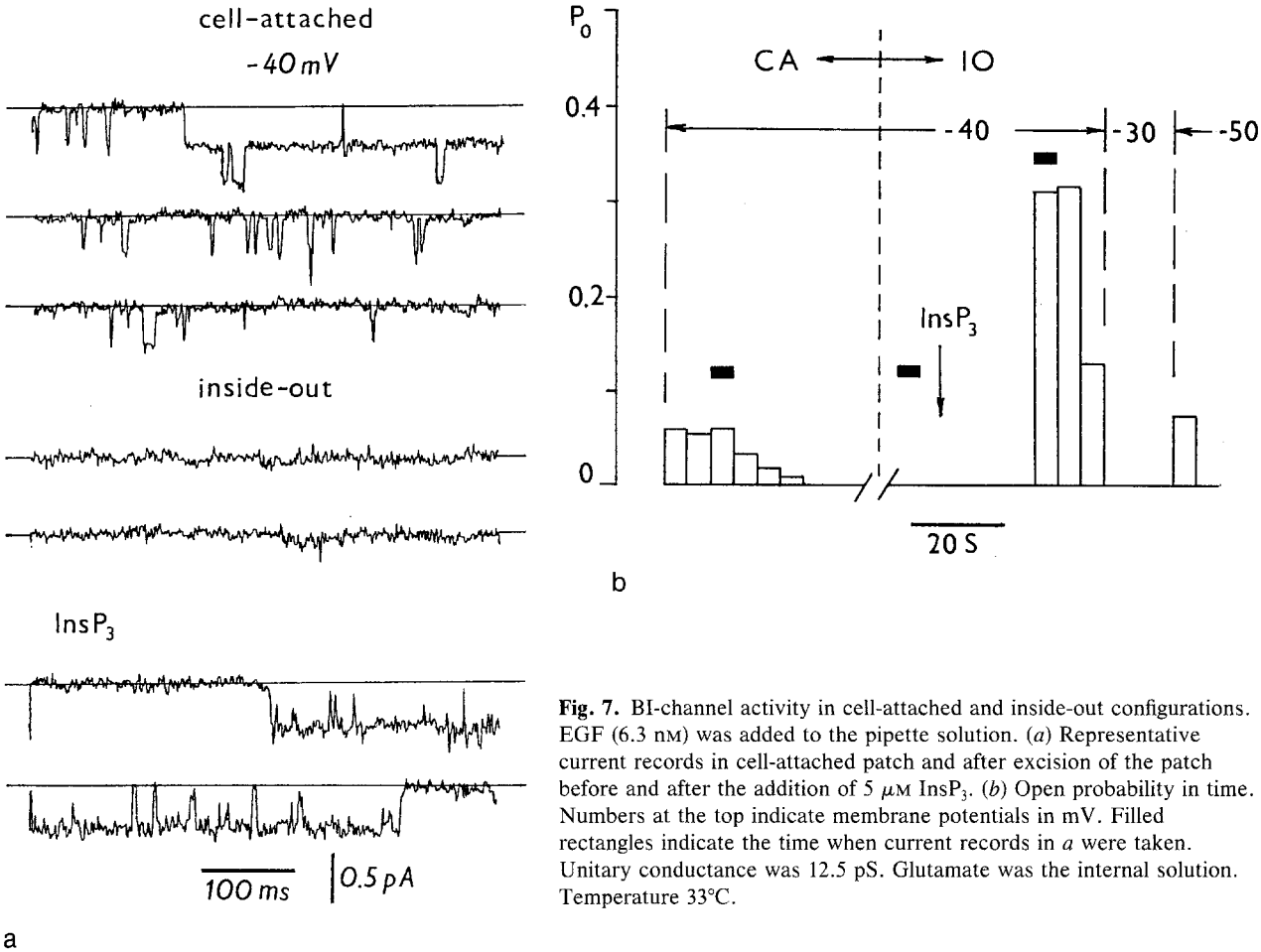


Fig. 7. BI-channel activity in cell-attached and inside-out configurations. EGF (6.3 nM) was added to the pipette solution. (*a*) Representative current records in cell-attached patch and after excision of the patch before and after the addition of 5 μM $InsP_3$. (*b*) Open probability in time. Numbers at the top indicate membrane potentials in mV. Filled rectangles indicate the time when current records in *a* were taken. Unitary conductance was 12.5 pS. Glutamate was the internal solution. Temperature 33°C.

slow part of the distribution seems to consist of more than one exponential component which cannot be characterized quantitatively with the data in hand. Similar results were obtained in two more experiments.

In addition to the four channel types described above we observed Ca^{2+}-permeable channels with mean conductances of 5.1 and 26 pS. Mechanisms of their control remain to be determined.

Discussion

SG channels seem to have the lowest conductance among Ca^{2+}-permeable channels; the nearest to them in this respect are voltage-gated Ca channels from *Paramecium* cilia incorporated into lipid bilayer (under comparable conditions their conductance was 2 pS) (Ehrlich et al., 1984).

The Ca^{2+} conductance of BI channels is a little higher than that of voltage-gated L channels (about 8 pS) (Hess, Lansman & Tsien, 1986). Two other types of voltage-gated channels were studied with the use of Sr^{2+} or Ba^{2+} as charge carriers (Kostyuk, Shuba & Savchenko, 1988); Sr^{2+} conductance of low-threshold inactivating and high-threshold inactivating channels was estimated to be 5.7 and 7 pS, respectively, which, again, is lower than conductance of BI channels (13.5 pS). On the other hand, judging by high values of reversal potential, this voltage-gated channels, especially the L type, are more selective for divalent cations against monovalent ones than the BI channels.

Channels with a conductance to 10 pS have been found in the membrane of mast cells (Kuno et al., 1989); however, they cannot be directly compared with BI channels because current measurements in that study were performed only with barium ions as the charge carrier.

In our recent cell-attached experiments on A431 cells (Mozhayeva et al., 1989*a*), we observed channels with conductances from 2 to 3.7 pS were responsive to EGF applied to the bath solution. They seemed to belong to the G and I types.

We believe that the channels found in our experiments mediate Ca^{2+} fluxes activated by EGF and other agonists. It should be noted, however, that the data in hand did not enable us to estimate precisely

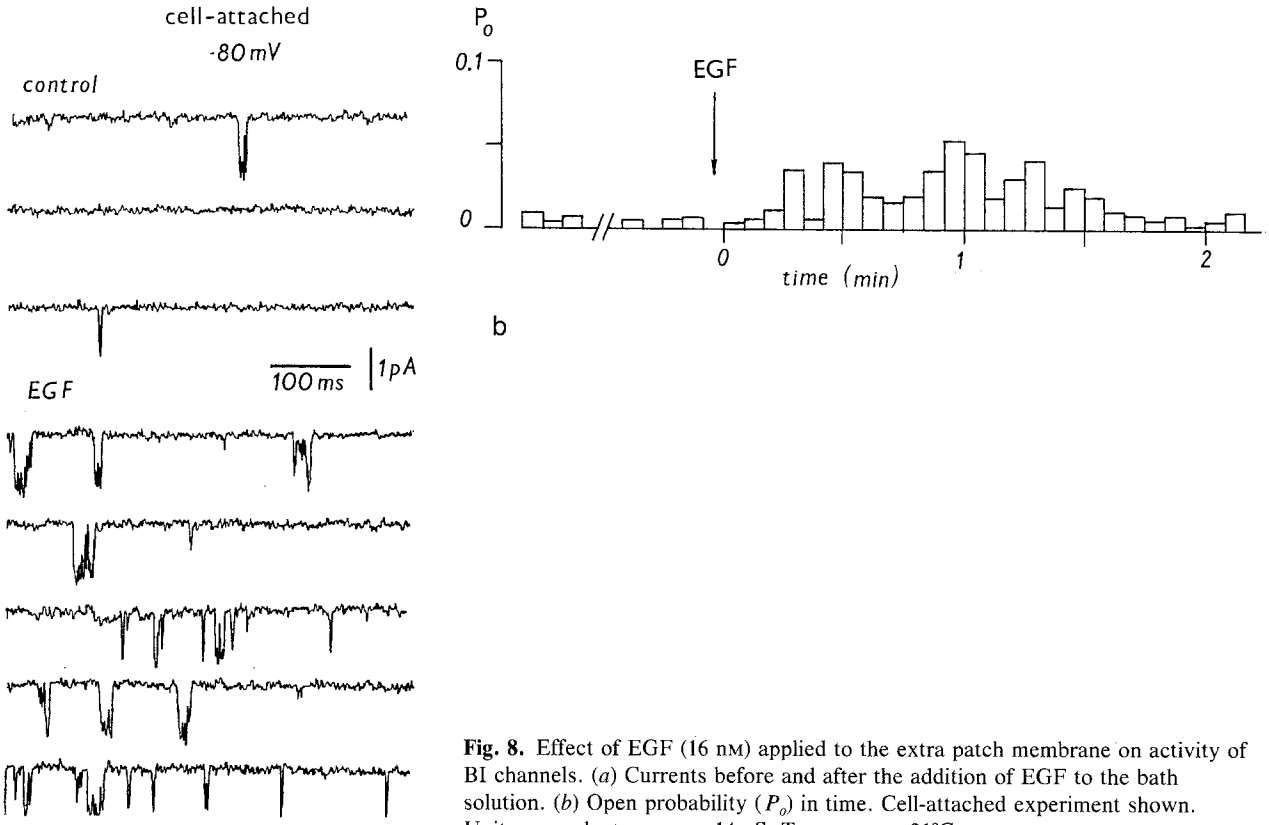


Fig. 8. Effect of EGF (16 nM) applied to the extra patch membrane on activity of BI channels. (*a*) Currents before and after the addition of EGF to the bath solution. (*b*) Open probability (P_o) in time. Cell-attached experiment shown. Unitary conductance was 14 pS. Temperature 31°C.

the amount of calcium flowing through these channels under physiological conditions because the measurements in our experiments were made at a high (105 mM) Ca^{2+} concentration. Single-channel and whole-cell experiments under different ionic conditions should be carried out to establish a link between electrophysiological data and those obtained in studies of Ca^{2+} fluxes and changes of cytoplasmic free calcium.

The responsiveness of SG and G channels to GTP nonhydrolyzable analogues suggests that G-proteins (Gilman, 1987; Neer & Clapham, 1988) are involved in the activation of both channel types. In accordance with this are the data that the treatment of A431 cells with aluminium fluoride, which is known to activate G-proteins, stimulates Ca^{2+} entry in these cells (Yu.A. Kuryshev, G.A. Savokhina, P.V. Avdonin and A.P. Naumov, *in preparation*).

There are precedents for involvement of G-proteins in the control of activity of Ca-permeable channels. Channels activated by internally applied GTPγS have been found in rat peritoneal mast cells (Matthews, Neher & Penner, 1989); the conductance of these channels with 95 mM $BaCl_2$ is 17 pS. Dihydropyridine-sensitive voltage-gated channels are upregulated by cholera toxin-sensitive G-protein (G_s) (Yatani et al., 1987). Pertussis toxin-sensitive G-proteins mediate either down- (Holz, Rane & Dunlap, 1986; Ewald, Sternweis & Miller, 1988) or upregulation (Hescheler et al., 1988) of voltage-gated channels in neuronal cells.

The presence of G-protein-dependent Ca-permeable channels in basophils and platelets has been suggested by the data that cholera toxin (a specific activator of G_s proteins) (Narasimhan et al., 1988) and aluminium fluoride (a nonspecific activator of G-proteins) (Bochkov et al., 1989) stimulate Ca^{2+} entry in basophils and platelets, respectively. It is possible that channels in these cells are analogous to SG and G channels.

IP_3 has been shown to open Ca-permeable channels in endoplasmic (sarcoplasmic) reticulum (Ehrlich & Watras, 1988), thereby, promoting Ca^{2+} release from it (Berridge & Irvine, 1989). There are procedents for the activating effect of $InsP_3$ on Ca-permeable channels in plasma membrane. $InsP_3$ has been reported to open Ca-permeable channels (7 pS with mono- and divalent cations) in T-lymphocytes (Kuno & Gardner, 1987) and to augment activity of voltage-gated channels from T-tubules of skeletal muscle (Vilven & Coronado, 1988).

$InsP_3$-dependent whole-cell Ca^{2+} currents and the associated rise of intracellular Ca^{2+} have been measured in mast cells (Penner, Matthews & Neher,

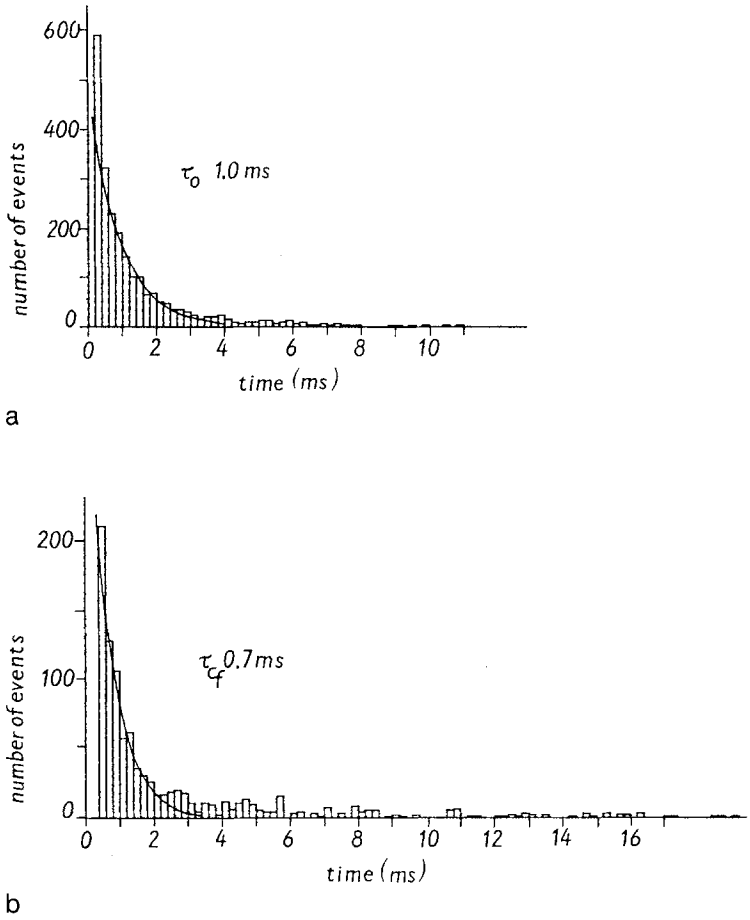


Fig. 9. Dwell-time histograms for a BI channel. (*a*) Open-time histogram. Filter was 1.5 kHz. The first two bins (200 μS) were discarded; the total number of events was 1642. Continuous curve represents a single exponential with a time constant of 1.0 msec. (*b*) Close-time histogram. Continuous curve represents an exponential with a time constant of 0.7 msec. Membrane potential was −80 mV.

1988; Matthews et al., 1989). The data suggest that these currents are mediated by channels which are specific for Ca^{2+} and have small (less than 1 pS) unitary conductance when the membrane is bathed by the normal external solution, i.e., containing only 2 mM or little more Ca^{2+}. It would be tempting to assume that $InsP_3$-sensitive, low-conductive channels in mast cells are identical with I or BI channels.

Stimulation of many cell-surface receptors initiates the hydrolysis of membrane-bound inositol lipid, thus, producing two second messengers: membrane-bound diacylglycerol and water-soluble $InsP_3$ (Berridge & Irvine, 1989). In A431 cells the production of $InsP_3$ has been shown to be stimulated by EGF, histamine, bradykinin and ATP (Hepler et al., 1987; Hosoi & Edidin, 1989). Thus, a requirement for activation of both I and BI channels is fulfilled in these cells, and one can assumed that both $InsP_3$-sensitive channels contribute to agonist-induced Ca^{2+} influx in A431 cells.

The agonist-induced $InsP_3$ release in A431 cells, as in many other cell types, is relatively short lived; therefore, the $InsP_3$-dependent component of Ca^{2+} influx mediated by I and BI channels is expected to be of the same or shorter duration. The more prolonged Ca^{2+} influx is mediated by other mechanisms, possibly, by SG and G channels.

None of the channels described here can be positively identified with any hitherto known Ca^{2+}-permeable channel type in other cells. It is unlikely that these channels are expressed only in A431 cells. It is more probable that these channels exist in other cell types too, but have not yet been found. There are, at least, two reasons for that. First, there are not many experimental studies aimed at searching for Ca-permeable channels in nonexcitable cells. Second, it seems to be a general rule that functional Ca^{2+}-permeable channels are not numerous in cell membranes. Thus, in A431 cells used in our experiments the vast majority of patches tested failed to display the activity of Ca^{2+}-permeable channels. Even G channels, which occurred more frequently than others, were observed in different series of experiments as rarely as in 6 to 2% of the patches tested. It is possible that the channels are under control of multiple mechanisms which occur in a

permissive state simultaneously but rarely. In such a case channels would be seen infrequently even if they are numerous in the membrane. Single-channel measurements under comparable conditions are necessary to establish if the variety of Ca^{2+}-permeable channel types found in A431 cells also occurs in other types of cells.

A number of recent studies have demonstrated that the intracellular calcium signal is a complicated array of Ca_i^{2+} changes, highly organized in space and time (*see* Jacob, 1990). It can be suggested that the variety of Ca^{2+}-permeable channels with different conductive properties and mechanisms of activation is necessary to generate the diversity of calcium signals depending on the type of agonist and the state of the cell.

We thank Dr. A.D. Sorkin for providing us with highly purified EGF and G.I. Ivanova and E.V. Kaznacheyeva for maintenance of the cell culture.

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Received 25 September 1990; revised 17 May 1991