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The effects of bradykinin on K^+ currents in NG108-15 cells treated with U73122, a phospholipase C inhibitor, or neomycin

Jan-Peter Hildebrandt, Timothy D. Plant & [1]Hans Meves

Physiologisches Institut der Universität des Saarlandes, D-66421 Homburg-Saar, Germany

1 Bradykinin has multiple effects on differentiated NG108-15 neuroblastoma × glioma cells: it increases Ins(1,4,5)P_3 production and intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, evokes a Ca^{2+} activated K^+ current ($I_{K(Ca)}$) and inhibits M current (I_M). We studied the effect of the aminosteroid U73122 and the antibiotic neomycin, both putative blockers of phospholipase C (PLC), on these four bradykinin effects.

2 Preincubation with 1 or 5 μM U73122 for 15 min partly suppressed Ins(1,4,5)P_3 generation and the increase in $[Ca^{2+}]_i$ induced by 1 μM bradykinin. U73122 10 μM caused total and irreversible inhibition. The inactive analogue U73343 was without effect.

3 Resting levels of Ins(1,4,5)P_3 were not affected. However, resting $[Ca^{2+}]_i$ was increased by 10 μM U73122, but not by U73343. Individual cells responded to 10 μM U73122 with a small increase in $[Ca^{2+}]_i$, followed in some cells by a large further rise.

4 Pretreatment of whole-cell clamped cells with 1 μM U73122 for 30 min reduced the bradykinin-induced $I_{K(Ca)}$ to a fifth of its normal size. To suppress it totally, a 7–12 min pretreatment with 5 μM U73122 was required. Again, U73343 was without effect.

5 U73122 and U73343 at concentrations of 5–10 μM irreversibly decreased the holding current (I_h) which at a holding potential of −30 or −20 mV mainly flows through open M channels. The decrease was often preceded by a transient increase.

6 M current (I_M) measured with 1 s pulses, was also decreased by 5–10 μM U73122 and U73343, but short applications of U73122 could cause a small increase. The bradykinin-induced inhibition of I_M was not affected by U73122.

7 Preincubation with 1 or 3 mM neomycin for 15 min did not affect Ins(1,4,5)P_3 generation and the increase in $[Ca^{2+}]_i$ induced by bradykinin. Pretreatment with 3 mM neomycin for about 20 min diminished the bradykinin-induced $I_{K(Ca)}$ to a fifth of its normal size.

8 The four main conclusions drawn from the results are: (a) U73122 suppresses bradykinin-induced PLC activation and $I_{K(Ca)}$, but not I_M inhibition. (b) This indicates that the transient outward current $I_{K(Ca)}$, but not the decrease of I_M in response to bradykinin, is mediated by PLC. (c) U73122 itself inhibits I_M and mobilizes Ca^{2+} from intracellular stores. (d) Externally applied neomycin is not an effective inhibitor of PLC-mediated signalling pathways in NG108-15 cells.

Keywords: U73122; neomycin; bradykinin; NG108-15 cells; M current; phospholipase C

Introduction

In NG 108-15 neuroblastoma × glioma hybrid cells, the non-apeptide bradykinin activates phospholipase C (PLC) resulting in the accumulation of inositol (1,4,5)-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DG) (Yano *et al*., 1985) and a transient increase in the cytosolic calcium activity ($[Ca^{2+}]_i$) (Reiser & Hamprecht, 1985). The rise in $[Ca^{2+}]_i$ activates K^+ channels leading to a transient hyperpolarization in current clamp (Reiser & Hamprecht, 1982) or to an outward current in voltage clamp (Brown & Higashida, 1988a). The M current, a non-inactivating K^+ current, is inhibited in NG108-15 cells by bradykinin (Brown & Higashida, 1988a,b; Schäfer *et al*., 1991). This latter effect is presumably not mediated by PLC, since neomycin, a putative inhibitor of phosphatidylinositol signalling pathways (Schacht, 1976), does not prevent M current inhibition by acetylcholine, a transmitter activating phosphatidylinositol signalling pathways as well, in NG108-15 cells transformed (by DNA transfection) to express m1 muscarinic receptors (Robbins *et al*., 1993). This indicates that PLC activation and M current inhibition are independent events, both triggered by activation of plasma membrane receptors coupled to G protein.

The present paper describes experiments with the PLC inhibitor U73122, a membrane permeable aminosteroid that blocks the phosphatidylinositol-specific PLC in a variety of cell types such as platelets, neutrophils and neuroblastoma cells (Bleasdale *et al*., 1990; Thompson *et al*., 1991). The mechanism of U73122 action is unclear at present. It may interrupt the coupling between G protein and PLC (Thompson *et al*., 1991) or decrease substrate availability for PLC (Vickers, 1993). Most investigators use U73122 at a concentration of 10 μM, but 10 or 100 times smaller concentrations have also been found to be effective (e.g. Jin *et al*., 1994). The close structural analogue U73343 does not affect PLC and can help to distinguish PLC-mediated from 'unspecific' U73122 effects.

Our results provide further evidence that PLC activation is responsible for the bradykinin-induced outward current but not for M current inhibition.

Methods

Cell culture

Mouse neuroblastoma × rat glioma hybrid cells, clone NG108-15, of passage numbers 14 to 50 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine. For the experiments, the cells were induced to differentiate by DMEM containing 1% foetal calf serum, 100 μM hypoxanthine, 16 μM thymidine,

[1] Author for correspondence.

10 μM prostaglandin E_1 and 50 μM isobutylmethylxanthine for 6–12 days before use.

Determination of Ins(1,4,5)P_3 accumulation

Cells cultured to confluency and differentiated were mechanically detached from the bottom of the culture flask, pelleted by centrifugation (1000 *g*, 5 min, room temperature) and resuspended in bath medium to a final protein concentration of 0.1–0.2 mg ml^{-1} (cf. Thompson *et al*., 1991). The bath medium contained (in mM): NaCl 136, $CaCl_2$ 2.6, KCl 2.4, $MgCl_2$ 1.2, glucose 10, tetraethylammonium chloride 5 and HEPES 15 (pH 7.4, adjusted with NaOH). Aliquots of cell suspension (200 μl) were transferred to 1.5 ml Eppendorf tubes. Assay tubes were placed in a water bath at 37°C for 10 min. Aminosteroid U73122 (1–10 μM), U73343 or neomycin (1–3 mM) were added to the cell suspensions 15 min before the cells were stimulated with 1 μM bradykinin. This bradykinin concentration has been previously shown to stimulate polyphosphoinositide breakdown in NG108-15 cells maximally (e.g. Campbell *et al*., 1990). Reactions were terminated by adding 200 μl of an ice-cold 1 M solution of trichloroacetic acid (TCA) to the cell suspensions. Assay tubes were vortexed and placed on ice for 15 min. Tubes were centrifuged for 10 min at 4°C at 8000 *g* to pellet the TCA-precipitates. Supernatants and pellets were processed separately. Protein pellets were resuspended in 3 M NaOH solution and protein concentration was measured (Bradford, 1976). Supernatants were extracted 3 times with excess water-saturated diethyl ether to remove residual TCA and subsequently neutralized by adding 65 mM $NaHCO_3$ solution. Ins(1,4,5)P_3 mass in this preparation was determined with a receptor binding assay procedure (Bentz & Hildebrandt, 1995).

Measurement of the intracellular free calcium concentration [Ca^{2+}]$_i$

Differentiated cells were brought into suspension as described above and loaded with 50 μM of the fluorescent calcium indicator dye indo-1 in its acetoxymethyl ester form (Grynkiewicz *et al*., 1985) for 15 min at 37°C in the dark. Cells were pelleted by centrifugation (1000 *g*, 2 min, room temperature), resuspended in fresh bath medium and incubated again for 15 min at 37°C in the dark. Cells were washed 3 times in fresh bath medium and finally resuspended in 3 ml bath medium in the cuvette of a Perkin-Elmer LS-5 spectrofluorimeter. The cell suspension was stirred and kept at 37°C throughout the measurement. Excitation and emission wavelengths were set at 332 nm and 400 nm, respectively. The fluorescence intensity curves were registered on a pen recorder and simultaneously stored in a microcomputer. Calibration of fluorescence signals was performed as described previously (Grynkiewicz *et al*., 1985; Shuttleworth & Thompson, 1989). For the experiments with PLC inhibitors, cells were incubated with the respective drugs for 15 min before being washed and resuspended in the cuvette. In these experiments, drugs were also present in the cuvettes during measurement and calibration.

For Ca^{2+} measurements on individual differentiated cells, cells were plated at a density of about 5×10^4 cells/35 mm diameter culture dish before differentiation. Measurements were performed with a slow-scan digital imaging system (T.I.L.L. Photonics GmbH, München, Germany) with Image-8 software (University of Saarland, Homburg, Germany). The cells were loaded with fura-2AM (Molecular Probes; added to the bathing solution at a concentration of 15 μM from a 5 mM stock solution in 20% Pluronic F-127/80% DMSO) for 10 min at 37°C. Fluorescence, produced by alternating excitation light at 358 and 375 nm, was collected from a rectangular field containing the soma. Image pairs were collected every 4 s. The background fluorescence from a region without cells or cell processes close to the cell was subtracted on-line. The exposure time to each wavelength was 100 ms.

Electrophysiological experiments

Plated and differentiated cells were voltage clamped in the whole-cell mode, with an EPC-7 amplifier (List Electronics) with partial series resistance compensation and pipettes with 2–4 MΩ resistance. The cell diameter was 30–60 μm. The experiments were done at room temperature (20°C) or at 30–35°C. For measuring I_M, 1 s pulses from a holding potential of −30 or −20 mV to potentials between −115 and −5 mV were used. The currents at the end of the 1 s pulses were measured and plotted against pulse potential V. Currents at $V < -70$ mV were taken as leakage currents, extrapolated to other potentials and subtracted from the total current to give $I_M(V)$.

Cells were continuously superfused with bath solution (flow rate 0.2–0.6 ml min^{-1} at 20°C, 0.6 ml min^{-1} at 30–35°C). The volume of the recording chamber was reduced to 0.77 ml by inserting a perspex ring (internal and external diameter 15 and 35 mm, respectively) into the culture dish. U73122, neomycin and bradykinin were applied via the bath.

The composition of the bath solution is given above. Na^+ currents were blocked by adding 0.2 μM tetrodotoxin. The composition of the pipette solution was (in mM): K aspartate 140, KCl 30, EGTA 3, $CaCl_2$ 0.8, $MgCl_2$ 0.15 and HEPES 10 (pH 7.2, adjusted with KOH). A pipette solution with K citrate, favourable for recording $I_{K(Ca)}$ (Robbins *et al*., 1992), was also used; it contained (in mM): K citrate 45, KCl 20, EGTA 3, $CaCl_2$ 1.0 and HEPES 40 (pH 7.2, adjusted with KOH).

Materials

U73122 (1-[6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-1H-pyrrole-2,5-dione; Bleasdale *et al*., 1990) and U73343 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione) were purchased from Biomol. The drugs were dissolved in chloroform to a final concentration of 5 mM and aliquotted into Eppendorf tubes. After evaporation of the solvent under nitrogen, tubes were stored at 4°C. On the day of an experiment, one aliquot was dissolved in water-free dimethylsulphoxide (DMSO) (Sigma) to a concentration of 10 mM. The final concentration of DMSO in the medium was 0.1% or less. For some of the electrophysiological experiments, U73122 and U73343 were frozen as 5 mM stock solutions in DMSO at −20 or −80°C for less than one week and were used without a difference in results. In these experiments, the final DMSO concentration was 0.2%. Bradykinin and neomycin sulphate were obtained from Sigma. Bradykinin was stored as a stock solution of 1 mM at −20°C, neomycin sulphate as a stock solution of 0.5 M at 4°C.

Statistical analysis

Wherever possible, averages ± s.e.mean are given. We used the two-tailed, unpaired *t* test to decide whether the difference between two average values was significant at the 5% level.

Results

Ins(1,4,5)P_3 accumulation in bradykinin-activated cells

Resting Ins(1,4,5)P_3 levels as measured with the receptor binding assay were 14.4 ± 1.6 pmol mg^{-1} protein (mean ± s.e.mean, $n = 6$) which resembles the levels determined previously by other authors (Donié & Reiser, 1991; Chueh & Kao, 1994; Smart & Lambert, 1996). Activation of the cells with 1 μM bradykinin for 15 s resulted in an accumulation of Ins(1,4,5)P_3 up to 50 pmol mg^{-1} protein (Figure 1a). Such an increase in Ins(1,4,5)P_3 upon stimulation of NG108-15 cells with saturating concentrations of bradykinin has been observed before (Campbell *et al*., 1990, and references therein; Kimura & Higashida, 1992). Preincubation of cells with the

aminosteroid U73122 in concentrations up to 10 μM did not affect resting levels of Ins(1,4,5)P_3, but partly (1 μM) or completely (10 μM) suppressed bradykinin-induced Ins(1,4,5)P_3 generation (Figure 1a). Preincubation of cells with U73343, the inactive analogue of U73122, did not affect either resting levels of Ins(1,4,5)P_3 or Ins(1,4,5)P_3 accumulation after stimulation of cells with bradykinin (Figure 1a). These results were obtained in cell suspensions with a protein concentration of 0.1–0.2 mg ml^{-1}. When cell suspensions with higher protein concentrations were used, we observed only a partial inhibition of bradykinin-induced Ins(1,4,5)P_3 generation by U73122, even when cells were preincubated with 10 μM of the amino-steroid (results not shown).

Preincubation of cells with neomycin resulted in an apparent increase in Ins(1,4,5)P_3 mass in the unstimulated cells that was dependent upon the neomycin concentrations (Figure 1b) and the length of the preincubation period. However, preincubation with neomycin in the concentration range 1–3 mM for 15 min did not affect the bradykinin-induced accumulation of Ins(1,4,5)P_3 (Figure 1b). This indicates that the offset in apparent Ins(1,4,5)P_3 mass in unstimulated cells by neomycin was caused by a substance that interfered with the receptor binding assay but was not Ins(1,4,5)P_3. Moreover, these data indicate that neomycin did not function as an effective inhibitor of PLC in NG108-15 cells when applied in the external medium.

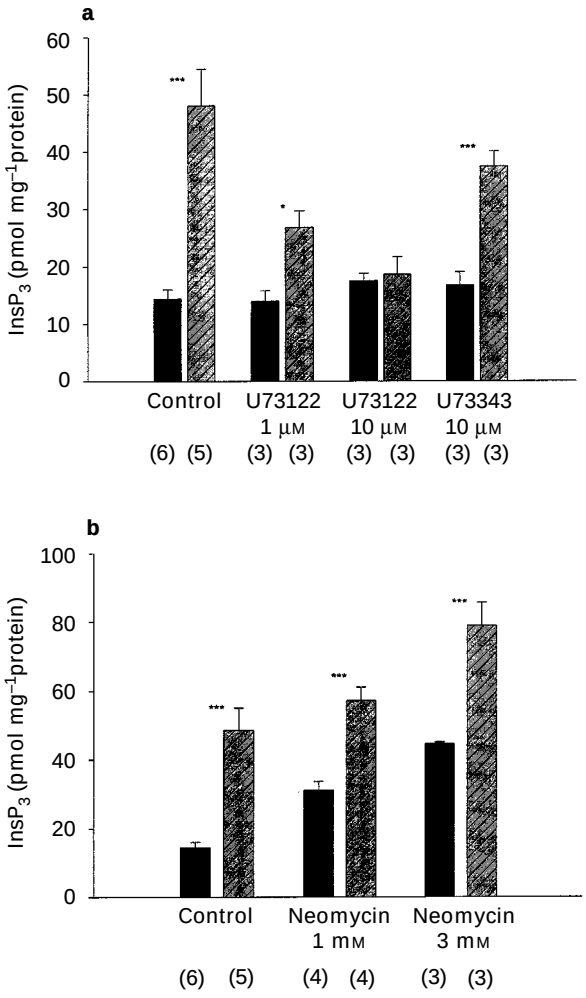

Figure 1 Ins(1,4,5)P_3 accumulation at rest (solid columns) and after application of 1 μM bradykinin (hatched columns) in control cells and in cells pretreated with U73122 or U73343 (a) or neomycin (b) for 15 min. Averages $\pm$s.e.mean from n (number in parentheses) experiments. Values marked *** and * are significantly different from values in bradykinin-free solution at the 0.1 and 5% level, respectively.

$[Ca^{2+}]_i$ measurements

The basal concentration of free calcium ions in the cytoplasm of isolated, indo-1 loaded NG108-15 cells was approximately 100 nM. When stimulated with 1 μM bradykinin, $[Ca^{2+}]_i$ rose steeply to peak values of 250–300 nM and declined thereafter to control values within 1 min (Figure 2a). Such a transient response in $[Ca^{2+}]_i$ to bradykinin with an apparent lack of any sustained calcium signal due to calcium influx has been observed before by other authors (Reiser & Hamprecht, 1985; Jin *et al*., 1994; Chueh & Kao, 1994). This transient calcium peak was partially suppressed when cells were preincubated with 1 μM U73122 for 15 min and was entirely absent when preincubation of cells was performed with higher concentrations of U73122 (Figure 2a). Washing for 20 min did not even partially reverse the effect of 10 μM U73122 applied for 15 min. Preincubation of cells with U73343 (1–10 μM) or neomycin (1–3 mM) did not affect the bradykinin-induced calcium signal (Figure 2b).

Preincubation of cell suspensions with 10 μM U73122 resulted in a slight increase in the unstimulated level of $[Ca^{2+}]_i$ (Figures 2a and 3), an effect that has been observed in other cell types as well as in NG108-15 cells (see Discussion). It was not seen with the inactive analogue U73343 (Figure 2b). The increase occurred within 1 min upon application of 10 μM U73122 (Figure 3a). Subsequent application of 10 μM bradykinin and 250 nM thapsigargin had no or little effect, suggesting that 10 μM U73122 had emptied the intracellar Ca^{2+} stores.

By using digital imaging with fura-2AM loaded cells, the behaviour of individual cells could be observed. The responses of individual cells varied considerably. All cells showed an initial small increase in $[Ca^{2+}]_i$ following application of 10 μM

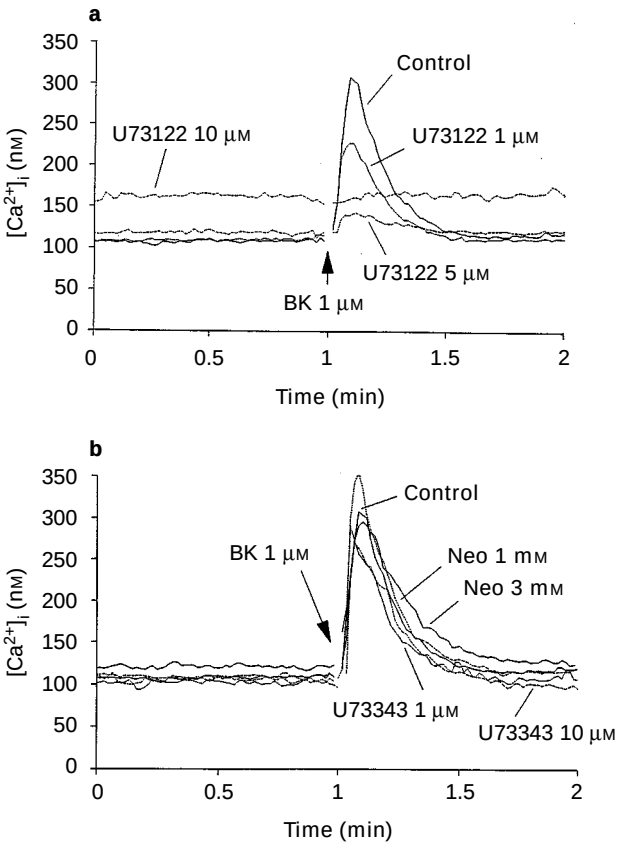

Figure 2 $[Ca^{2+}]_i$ in suspensions of NG108-15 cells at rest and after application of bradykinin (BK). (a) Control cells and cells pretreated with U73122 (1, 5 or 10 μM) for 15 min. (b) Control cells and cells pretreated with U73343 (1 or 10 μM) or neomycin (Neo, 1 or 3 mM) for 15 min. Traces represent means of n experiments on independent batches of cells; $n=5$ for controls, $n=6$ for 10 μM U73122, $n=2$ for 10 μM U73343, $n=3$ or 4 for all others.

U73122. In some cells this small increase was followed by a fast spike-like rise (Figure 3b, record i), in others by a medium-sized secondary response (Figure 3b, record ii). From a total of 17 cells, 5 responded with a fast spike-like rise as in record (i), 7 with a medium-sized signal as in record (ii) and 5 only with a very small and slow increase as in record (iii). No correlation between Ca^{2+} signal and cell diameter was found. $[Ca^{2+}]_i$ started to decline again before application of the drug ended. The Ca^{2+} signal in record (i) of Figure 3b was similar in size to the Ca^{2+} signal elicited by a bath solution with 50 mM KCl.

These results indicate that the bradykinin-induced calcium signals are directly dependent upon $Ins(1,4,5)P_3$ generation by PLC. Attenuation of PLC function by U73122, but not by exogenous neomycin, reduced the bradykinin-induced calcium release from intracellular stores into the cytosol.

Transient outward current

Normally, the holding current at a depolarized holding potential (−45 to −20 mV) responds to bradykinin with a transient increase followed by a longer lasting decrease (Brown & Higashida, 1988a,b; Schäfer *et al*., 1991). The initial increase is extremely variable in occurrence and size and desensitizes rapidly. It can be blocked by a mixture of apamin (0.1 μM) and charybdotoxin (10 nM) (Robbins *et al*., 1992) and is interpreted as a K^+ outward current activated via the PLC-IP_3-$[Ca^{2+}]_i$ pathway (Brown & Higashida, 1988b; Shimahara *et al*., 1990). The longer lasting decrease reflects inhibition of I_M, originally thought to arise from the activation of protein kinase C via the PLC - diacylglycerol (DG) pathway (Brown & Higashida, 1988b).

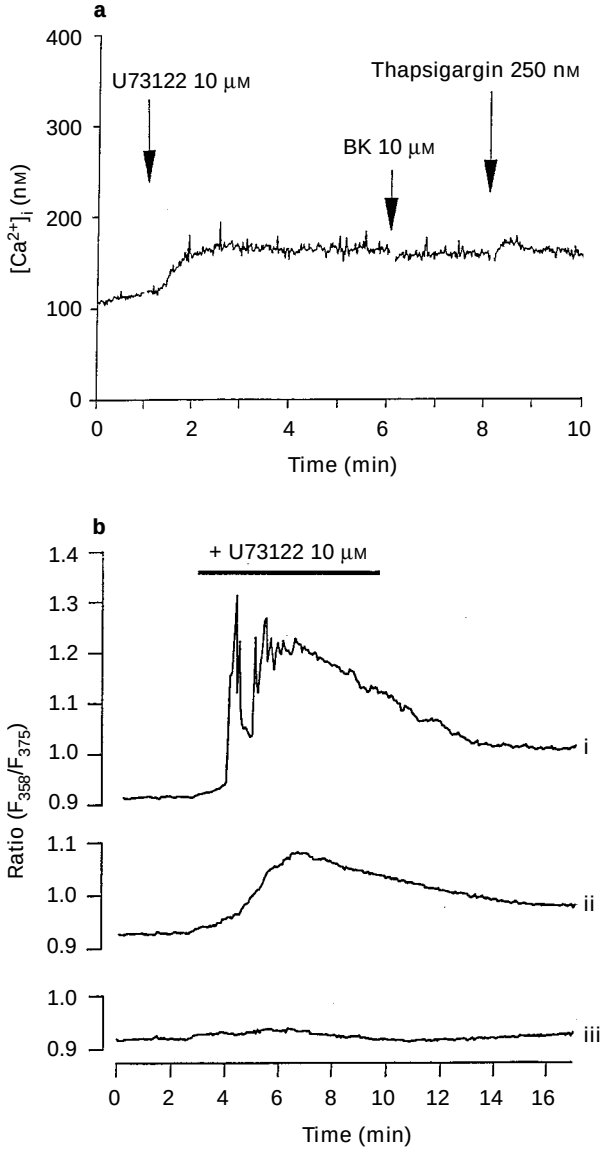

Figure 3 Rise in $[Ca^{2+}]_i$ upon application of 10 μM U73122 in a cuvette experiment (a) and in individual cells (b). In (a), application of 10 μM U73122 is followed by application of 10 μM bradykinin (BK) and 250 nM thapsigargin. (b) Change of fluorescence ratio F_{358}/F_{375} in three different cells (diameter 33, 28, 19 μm for i, ii, iii). Temperature 37°C in (a) and 34°C in (b).

The records in Figure 4a, b are from two cells treated with 1 μM U73122 and show that this treatment did not abolish the biphasic response to bradykinin, either at 20°C or at 34°C. Results from several experiments are presented in Table 1. Under control conditions with K aspartate as pipette solution at 30–35°C (line a) and at 20°C (line f), more than 50% of the cells responded to 1 μM bradykinin with an initial transient outward current. Its average size exceeded 200 pA. (At the higher temperature, the outward current was often preceded by a small current decrease as in Figure 4b, making the response to bradykinin triphasic rather than biphasic). With K citrate in the pipette (line i), the initial outward current occurred more often (cf. Robbins *et al*., 1992), albeit with a smaller amplitude. Pretreatment with 1 μM U73122 for 8–13 min at 30–34°C failed to affect the bradykinin response (line b), but longer pretreatment (25–30 min) at 20°C diminished the average size of the outward current to about a fifth (line g). Treatment with 5 μM U73122 for 7–12 min at 30–34°C totally abolished the bradykinin-induced outward current (line c). The cells responded only with a decrease in holding current (see Figure 7a). The effect of 5 μM U73122 seemed to be reversible, since the transient outward current recovered, when the cell was washed for 4–8 min with normal bath between U73122 treatment and bradykinin test (line d). The same was observed with 10 μM U73122 in 3 of 10 experiments at 20°C (line j). In one of these three experiments the transient outward current was unusually large (Figure 4c). The inactive analogue U73343 at a concentration of 5 μM had no effect on occurrence and size of the bradykinin-induced outward current (line e).

M current

U73122 at a concentration of 5 μM or more affected the M current. Brief treatment often slightly augmented I_M (see below); longer treatment decreased it (Figure 5). At $V = -35$ mV and 20°C, the inhibition by 5 μM U73122 was 43% at 15 min and 89% at 34 min (Figure 5c). At 30–35°C, the inhibition by 5 μM U73122 was more rapid, being 49.6±5.6% ($n = 5$) at 4–6 min. An experiment with 10 μM U73122 at room temperature is shown in Figure 5d. The inhibition after 5 min of treatment with 10 μM U73122 was 63% at V = −35 mV. In a further experiment with 20 μM U73122 at 20°C, the inhibition was similar, namely 67% at 11 min and V = −35 mV. The decrease of I_M could not be reversed by 10–20 min washing. The inactive analogue U73343 also inhibited I_M; at 30–35°C the inhibition by 5 μM U73343 applied for 7–12 min was 34.6±9.1% ($n = 7$).

The slight increase in I_M often produced by brief treatment with 5 or 10 μM U73122 is illustrated in Figure 6. Both the holding current at −30 mV and the current at the end of the 1 s pulse are slightly larger in 5 μM U73122. In four experiments at 30–35°C the average increase of I_M at −35 mV was 14.2±5.0% at 3 min. With repeated measurements on the same cell, a small increase at 4 min and a decrease at 10 min was seen. In a different type of experiment (Figure 6B), I_M was measured shortly after a 5 min application of 5 μM U73122. Under these conditions, $I_M(V)$ was slightly increased. ΔI_M at −35 mV amounted to 28%. In 5 experiments of this type at room temperature, ΔI_M at −35 mV was on average 29.8±9.0%.

The increase in holding current upon application of 5 or 10 μM U73122 occurred in about 50% of the cells investigated at 20 or 30–35°C. The increase was always transient, and was

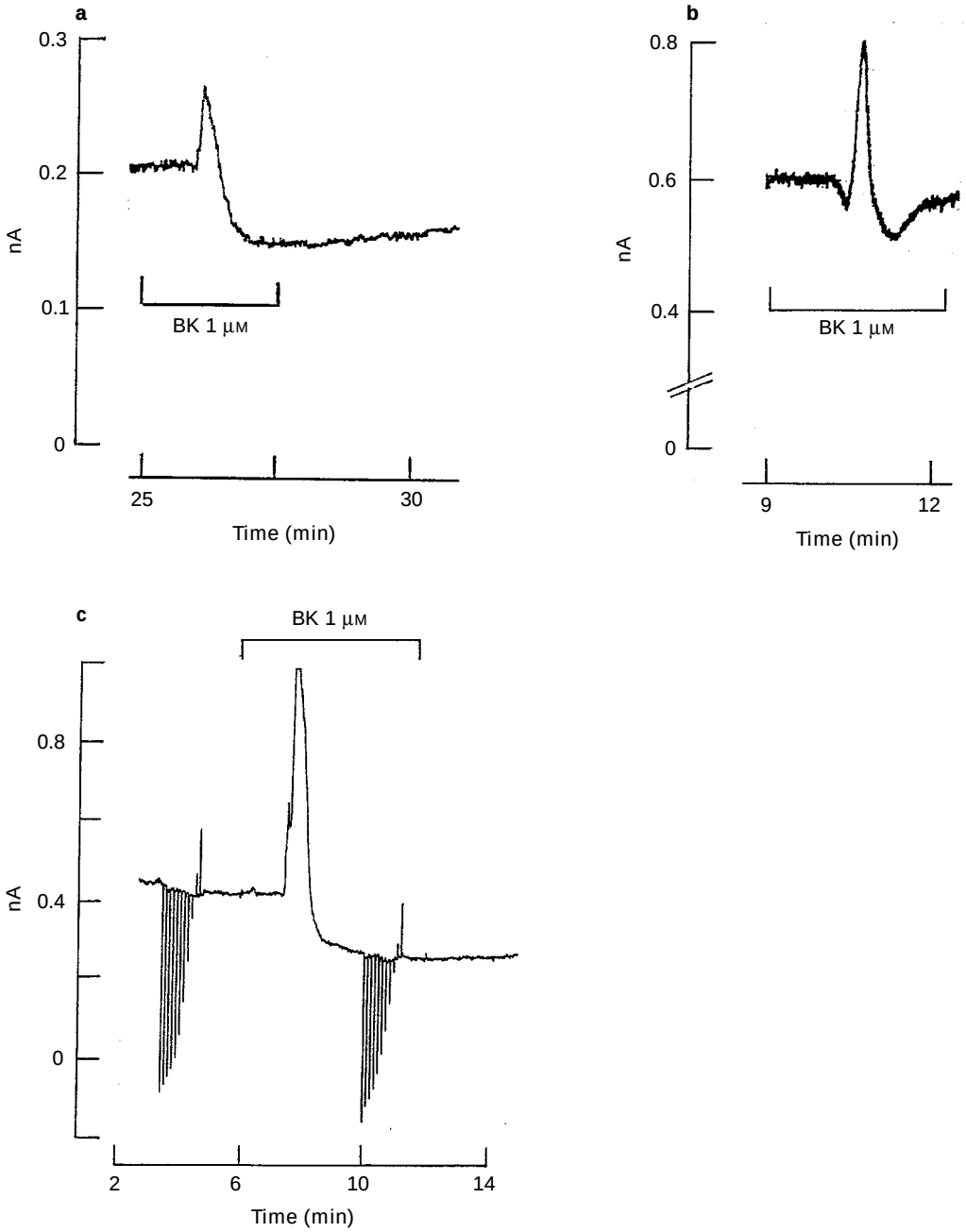

Figure 4 Action of 1 μM bradykinin on holding current following prolonged treatment with 1 μM U73122 (a, b) or brief treatment with 10 μM U73122 and wash (c). (a) Cell treated 25 min with 1 μM U73122 before bradykinin application; temperature 20°C. (b) Cell treated 9 min with 1 μM U73122 before bradykinin application; temperature 34°C. (c) Cell treated 5 min with 10 μM U73122 and washed 6 min before bradykinin application. Two pulse families for measuring M current; temperature 20°C. Abscissa scale gives time in bath with 1 μM U73122 or time of wash. Holding potential −20 mV. Pipette solution K aspartate in (a) and (b), K citrate in (c). Cell diameter 53 μM in (a), 56 μm in (b), 66 μm in (c).

followed by a faster or slower decrease. The other 50% of cells responded only with a decrease (Figure 7a). The initial increase was also observed in 4 out of 9 cells in which $I_{K(Ca)}$ was blocked by 0.1 μM apamin and 10 nM charybdotoxin. However, application of 10 μM of the structural analogue U73343, caused only a decrease of holding current ($n=4$).

U73122 did not abolish or significantly diminish the inhibition of I_M by 1 μM bradykinin. In experiments at room temperature, bradykinin inhibited I_M at −35 mV by $42.8\pm6.3\%$ ($n=12$) in control cells and by $38.6\pm13.4\%$ ($n=5$) in cells treated with 1 μM U73122 for 27–32 min. In later experiments at 30–35°C 5 μM U73122 decreased I_M, which was then further decreased by 5 μM U73122 + 1 μM bradykinin (Figure 7a,b). At −35 mV and 30–35°C, inhibition of I_M by 1 μM bradykinin was $64.9\pm6.9\%$ ($n=9$) in control cells and $50.3\pm19.0\%$ ($n=3$) in cells treated with 5 μM U73122 for 8–9 min.

Experiments with neomycin

Further experiments were done with neomycin, an aminoglycoside antibiotic which has often been used as an inhibitor of PLC-mediated signalling processes. Pretreatment for 18–23 min with 3 mM neomycin significantly reduced the size of the transient outward current that occurs in response to 1 μM bradykinin (Table 1). Pretreatment with 1 mM neomycin for 15–28 min ($n=10$) or 4 mM neomycin for 5 min ($n=7$) did not significantly reduce the bradykinin response (data not

Table 1 Transient outward current in response to 1 μM bradykinin

	Temperature (°C)	*Pipette solution*	*PLC inhibitor*	*Cells showing transient outward current* (%)	*Average size of outward current* (pA)
a	30–35	K aspartate	–	7/13 = 54	207 ± 47
b	30–35	K aspartate	U73122 1 μM for 8–13 min	3/7 = 43	156 ± 52
c	30–35	K aspartate	U73122 5 μM for 7–12 min	0/10 = 0	0*
d	30–35	K aspartate	U73122 5 μM for 5–6 min, then wash	2/4 = 50	240 ± 120
e	30–35	K aspartate	U73343 5 μM for 7–12 min	4/6 = 67	221 ± 82
f	20	K aspartate	–	6/10 = 60	240 ± 68
g	20	K aspartate	U73122 1 μM for 25–30 min	6/15 = 40	52 ± 15*
h	20	K aspartate	Neomycin 3 mM for 18–23 min	5/11 = 45	61 ± 16*
i	20	K citrate	–	11/13 = 85	91 ± 23
j	20	K citrate	U73122 10 μM for 5 min, then wash	3/10 = 30	300 ± 144*
k	20	K citrate	Neomycin 3 mM for 19–20 min	7/9 = 78	21 ± 6*

Holding potential −30 or −20 mV. *Value significantly different from respective control value in line a, f or i.

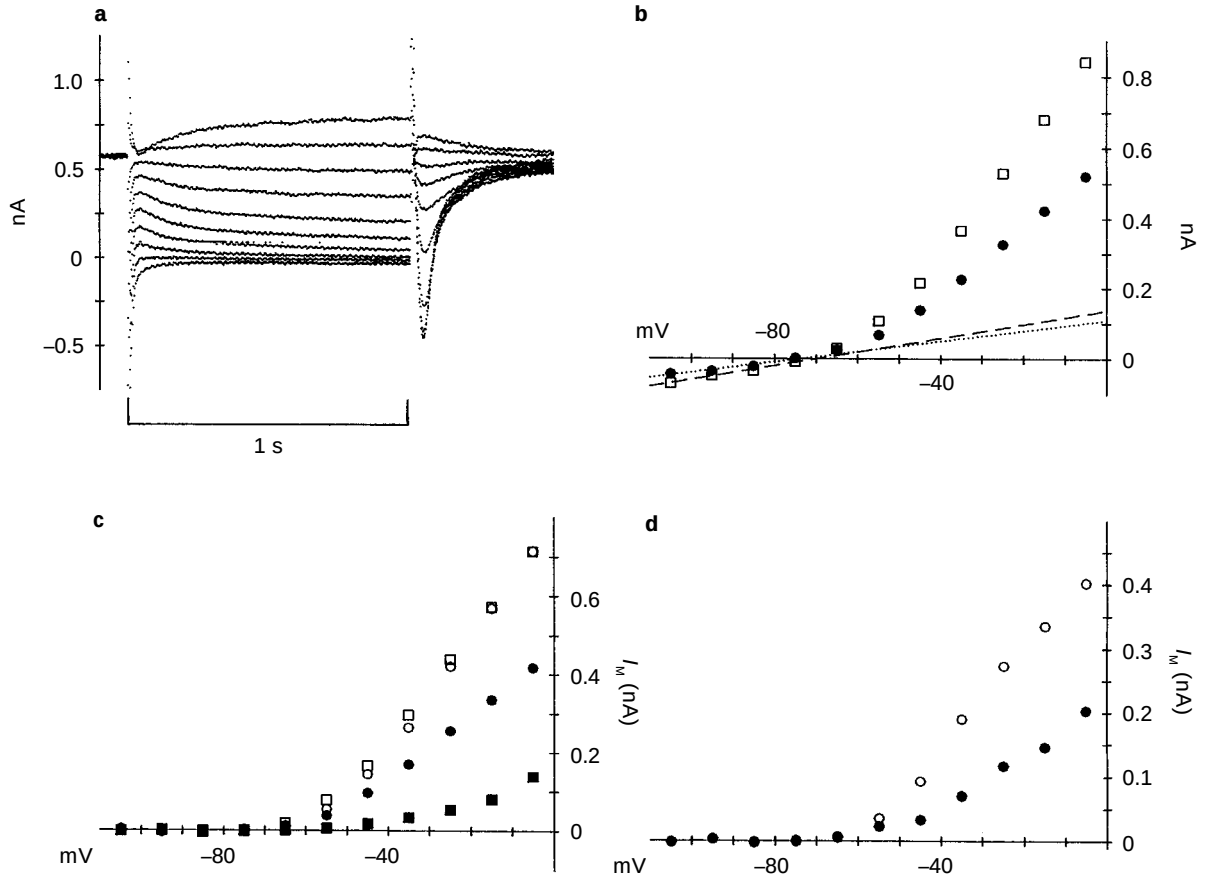

Figure 5 Block of M current (I_M) by 5 and 10 μM U73122. Holding potential −20 mV. (a) Currents elicited by 1 s pulses to potentials between −95 mV (lowermost record) and −5 mV (uppermost record) in 10 mV steps. (b) □. Currents at end of 1 s pulses in (a) plotted against pulse potential; (●) same for a pulse family recorded after 15 min treatment with 5 μM U73122; straight lines fitted to the points at $V < -70$ mV to give leakage conductance g_l (dashed line with $g_l = 2.0$ nS for (□), dotted line with $g_l = 1.5$ nS for (●)). (c) M current (= total current − leakage current) vs pulse potential. (□) Control (pulse family in (a)); (○) another control (5 min before (□)); (●) 15 min in 5 μM U73122; (■) 34 min in 5 μM U73122. (d) M current vs pulse potential for another cell in control (○) and after 5 min treatment with 10 μM U73122 (●). Pipette solution K aspartate in (a)–(c), K citrate in (d). Cell diameter 60 μm in (a)–(c) and 53 μm in (b). Temperature 20°C.

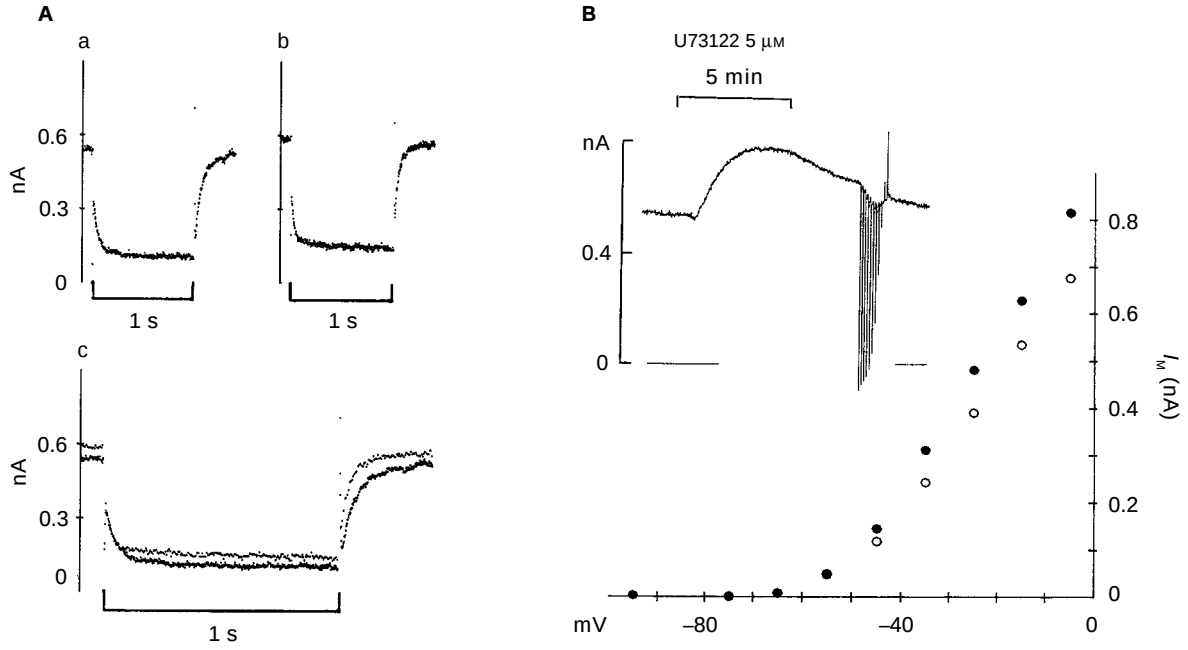

Figure 6 Small increase of M current (I_M) produced by short (3 or 5 min) treatment with 5 μM U73122. (**A**) The cell was held at -30 mV to activate I_M and stepped to -45 mV for 1 s to deactivate it. Current in control (a) and after 3 min treatment with 5 μM U73122 (b). The two currents superimposed in (c). Pipette solution K aspartate; temperature 33°C; cell diameter 68 μm. (**B**) I_M(V) of another cell before (○) and after (●) 5 min treatment with 5 μM U73122. Inset: change in holding current and pulse family. Pipette solution K citrate; temperature 20°C; cell diameter 60 μm.

shown). Neomycin applied for 16 min at a concentration of 1 mM also did not affect I_M ($n=3$) (see Figure 7c). Neomycin, 3 mM, applied for 11 min at 30–35°C, produced $31.3\pm3.9\%$ inhibition of I_M at -35 mV ($n=3$), which was partially reversible. Moreover, 1–2 mM neomycin did not abolish the bradykinin-induced M current inhibition (Figure 7c). At room temperature, I_M inhibition by 1 μM bradykinin in cells pretreated with 1–2 mM neomycin for 18–21 min was on average $28.6\pm14.8\%$ ($n=4$), which is not significantly different from the control value ($42.8\pm6.3\%$ $n=12$, see above).

Discussion

We have convincingly demonstrated that extracellularly applied U73122 inhibited bradykinin-induced Ins(1,4,5)P_3 generation and the transient increase in $[Ca^{2+}]_i$ in NG108-15 cells. This indicates that U73122 acts as a membrane-permeable inhibitor of phospholipase C (PLC) in these cells. Short incubation periods with U73122 seem to allow the cells to recover from the PLC-inhibition as found in the electrophysiological experiments. However, longer lasting preincubation of cells (15 min) with 5 or 10 μM U73122 caused an irreversible block of PLC (85 or 100%, respectively) that could not be relieved by washing cells in drug-free medium. However, comparison of such cuvette experiments and electrophysiological experiments must be made with caution. As pointed out by Civan *et al.* (1993), whole-cell patch clamp studies are commonly conducted on the largest cells which constitute a minority of the entire cell population: 'Therefore, chemical measurements of all the cells cannot be readily correlated with the electrophysiological properties of the minority-cell population.' Nevertheless, with regard to the effects of U73122 we found a certain coincidence in the behaviour of single cells in the electrophysiological experiments, in calcium measurements in single cells as well as in cell suspensions and in biochemical assays of inositol phosphate generation. One of these points is that U73122 seems to have effects on NG108-15 cells that cannot readily be explained by inhibition of PCL alone, especially when the aminosteroid is administered in higher concentrations and for prolonged periods of time. As indicated by patch-clamp experiments, long lasting applications of 5 or 10 μM U73122 decreased holding current and I_M. Preincubation of cells with 10 μM U73122 induced an increase in the $[Ca^{2+}]_i$ baseline in cell suspensions as well as in single cells (Figure 3) that has also been observed by others on NG108-15 cells (Jin *et al.*, 1994) and other cells (Smallridge *et al.*, 1992; Willems *et al.*, 1994). This effect seems to involve release of calcium from intracellular stores perhaps accompanied by changes in the plasma membrane permeability to calcium. The latter is indicated by the sustained elevation of $[Ca^{2+}]_i$ with U73122 (10 μM) not observed with bradykinin.

On the other hand, treatment of cells with U73122 for shorter periods of time and subsequent washing for 4–8 min was often not sufficient to block the bradykinin-induced $I_{K(Ca)}$ completely. This is paralleled by the observation in cell suspensions that at least 5 μM U73122 was required to suppress bradykinin-induced accumulation of Ins(1,4,5)P_3. For the electrophysiological experiments, therefore, we chose to incubate cells with 5 μM U73122 for 7–12 min and then switch to 5 μM U73122 + 1 μM bradykinin. Under these conditions, U73122 blocked the bradykinin-induced potassium outward current entirely (Table 1 line c), a finding that is consistent with the notion that this portion of the current response is mediated by phospholipase C action, inositol phosphate accumulation and elevations in $[Ca^{2+}]$ in these cells. However, the irreversible reduction in I_M observed in U73122-treated cells (Figure 5) was not a result of PLC-inhibition, because the inactive analogue U73343 had the same effect. U73122 and U73343 have been shown to inhibit voltage-gated calcium currents in smooth muscle cells (Macrez-Leprêtre *et al.*, 1996), so, it seems possible that they may also directly block M-channels in NG108-15 cells. The reason for the small and transient increase in I_M which often preceded its decrease by 5 or 10 μM U73122 (Figure 6) remains unknown.

U73122 did not affect the inhibition of M-current by bradykinin, indicating that PLC is not likely to be involved in agonist-mediated inhibition of I_M. The same conclusion has been reached by Robbins *et al.* (1993) with regard to the ACh-

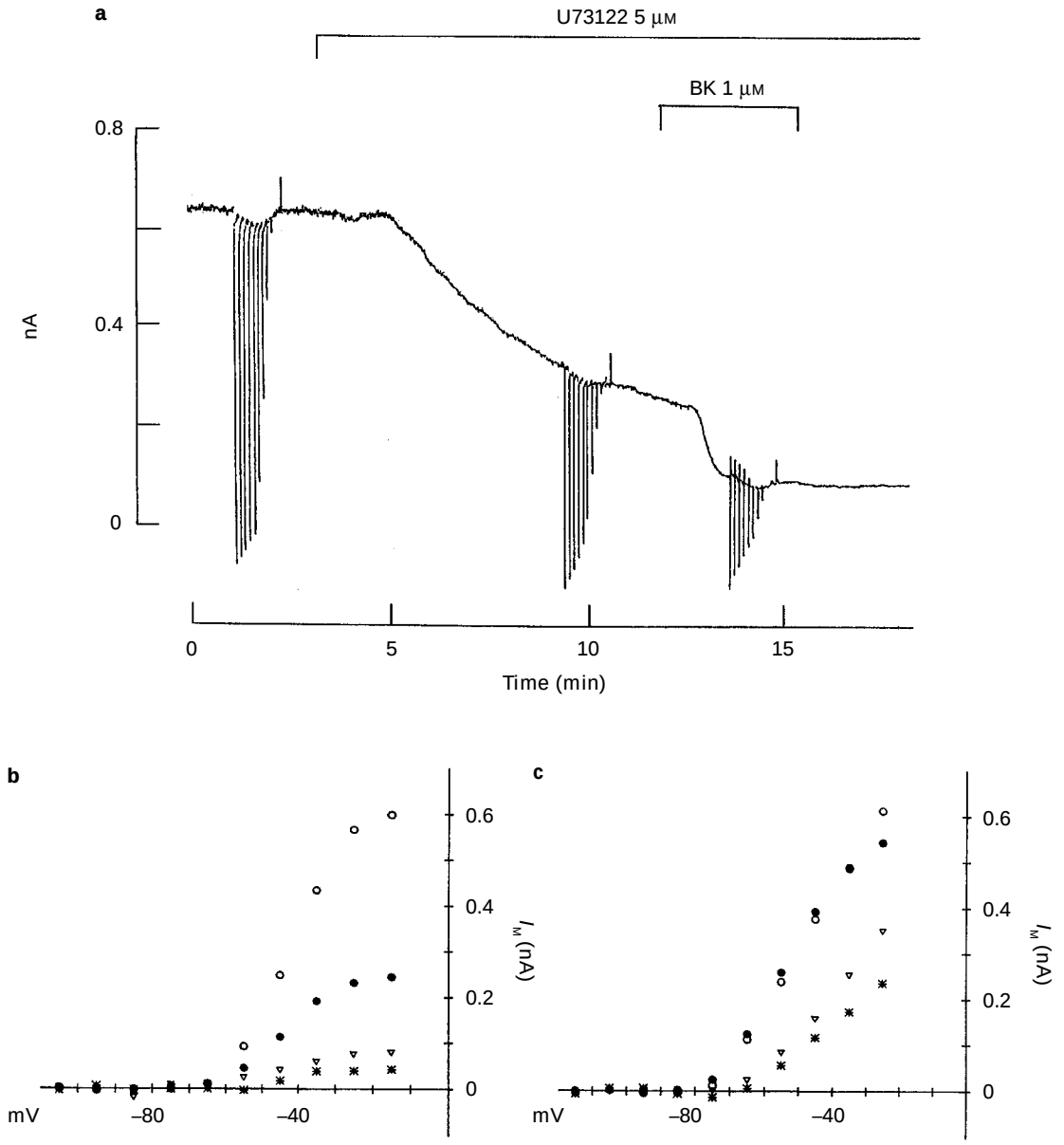

Figure 7 Inhibition of M current (I_M) by 1 μM bradykinin in the presence of 5 μM U73122 (a, b) or 1 mM neomycin (c). (a) Pen record showing solution sequence, changes in holding current and pulse families. (b) I_M (V) plot for the same cell in control (○), in 5 μM U73122 for 6 min (●), in 5 μM U73122 + 1 μM bradykinin (*) and again in 5 μM U73122 for 6 min (▽). Holding potential −20 mV; cell diameter 53 μm; temperature 34°C. (c) I_M(V) plot for another cell in control (○), in 1 mM neomycin for 16 min (●), in 1 mM neomycin + 1 μM bradykinin (*) and again in 1 mM neomycin for 10 min (▽). Holding potential −30 mV; cell diameter 64 μm; temperature 20°C. Pipette solution K aspartate in both cells.

induced inhibition of I_M. The agonist-mediated inhibition of I_M seems to result from a direct interaction between G protein and M channel or from the effects of an as yet unidentified second messenger molecule (cf. discussion in Robbins *et al.*, 1993). The latter possibility became more likely recently, since Selyanko *et al.* (1995) showed an inhibition of M channel activity in cell-attached membrane patches when agonists were applied to the medium surrounding the NG108-15 cells. A possible candidate for such a diffusible intracellular messenger molecule could be cyclic ADP-ribose (cADPR) (Higashida *et al.*, 1995; 1996).

The triphasic bradykinin effect in Figure 4b indicates M current inhibition starting before $I_{K(Ca)}$ activation (for a similar observation with ACh, see Figure 6 of Robbins *et al.*, 1993). This reinforces the case for separating the transduction pathways for $I_{K(Ca)}$ activation and I_M inhibition.

We were unable to suppress or reduce the bradykinin-induced Ins(1,4,5)P_3-accumulation and the $[Ca^{2+}]_i$ transient by pretreatment of cells with 1 or 3 mM neomycin for 15 min. However, in two series of experiments, treatment with 3 mM neomycin for 18–23 or 19–20 min reduced the bradykinin-induced transient outward current to a fifth of its normal size but failed to abolish it. These findings were surprising since extracellularly applied neomycin has often been used as a convenient inhibitor for phospholipase C-mediated signalling events. It has been shown to inhibit agonist-induced inositol phosphate formation in fibroblasts and pituitary gonadotrophs (Carney *et al.*, 1985; Zheng *et al.*, 1994) and agonist-induced calcium signals in pancreatic acinar cells (Saito *et al.*, 1996). Robbins *et al.* (1993) were able to suppress the ACh-induced transient outward current of NG108-15 cells by 1 mM neomycin in five out of six cells. However, negative results have also been published. In rat glioma cells, Reiser *et al.* (1990) found no influence of up to 2 mM neomycin on the bradykinin-induced hyperpolarization. Two recent studies on smooth muscle cells found no effects of neomycin on agonist-induced phosphatidylinositol hydrolysis and little effect on the $[Ca^{2+}]_i$ increase (Cox *et al.*, 1996; Sipma *et al.*, 1996).

The positively charged aminoglycoside neomycin blocks phosphatidylinositol signalling by forming an electroneutral complex with negatively charged membrane lipids, preferentially with phosphatidylinositol (4,5)-bisphosphate (PIP_2), thereby rendering it inaccessible to phospholipsae C (Schacht, 1976). Since many studies show clearly that intracellular application of neomycin inhibits phospholipase C action on PIP_2, it seems likely that the difference in the efficacy of extracellular neomycin in inhibiting PLC-mediated signalling in various cell types results from differences in plasma membrane permeabilities for this antibiotic. Whilst neomycin exerts its inhibitory effects on inositol phosphate generation in cells by blocking PIP_2 hydrolysis, some of the effects of neomycin on plasma membrane currents may actually be based on direct interactions with ion transport systems. Neomycin blocks voltage-sensitive calcium channels in brain nerve endings from the extracellular side (Canzoniero *et al*., 1993) and is able to block calcium-activated K^+ channels in rat brain synaptosomes by binding to the channels from the cytoplasmic side with a K_d of 195 μM (Nomura *et al*., 1990). Our observation that long-lasting application of 3 mM neomycin in the extracellular medium reduced the transient outward current to a fifth of the control value could be explained by similar actions of neomycin on NG108-15 cells. It seems possible that neomycin molecules slowly permeated through the membrane and blocked $I_{K(Ca)}$ directly.

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