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THE CYCLIC AMP-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT SELECTIVELY ENHANCES CALCIUM CURRENTS IN RAT NODOSE NEURONES

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SUMMARY

1. The whole-cell variation of the patch clamp technique was used to study the effect of the purified catalytic subunit of the cyclic AMP-dependent protein kinase (A kinase catalytic subunit: AK-C) on the calcium current components of acutely dissociated rat nodose ganglion neurones.

2. The transient low-threshold calcium current component (T) was stable during whole-cell recording. In contrast, currents containing the transient high-threshold (N) and slowly inactivating high-threshold (L) current components declined steadily after stabilization of the currents during the first 5–7 min of recording. When AK-C was included in the recording pipette at physiological concentrations (50 μg/ml, approximately 1 μM), currents containing the N- and L-components increased in magnitude beginning 7–9 min after patch rupture, but there was no effect on the isolated T-current. The current–voltage relation of the T-current component was similar to controls, but the current–voltage relation for the N- and L-current components was shifted slightly to more depolarized clamp potentials (V_c), approximately 10 mV.

3. The effect of AK-C on currents containing the N- and L-currents was concentration dependent. There was no effect of 0·1 μg/ml AK-C, the lowest concentration tested. Currents evoked from holding potentials (V_h) = −80 mV increased 5–10% during a 20 min recording in the presence of 1 μg/ml AK-C and 30–35% in the presence of 50 μg/ml AK-C. In contrast, currents evoked from V_h = −40 mV increased 5–10% in the presence of either 1 or 50 μg/ml AK-C. The increase in current magnitude was associated with an increased rate of current inactivation and was evident particularly in currents evoked from V_h = −80 mV.

4. These effects were blocked by prior incubation of AK-C (1 μg/ml) with a specific peptide inhibitor (protein kinase inhibitor peptide, PKIP; 0·2 mg/ml).

5. We evoked calcium currents using very long (1 s) voltage commands and modelled the traces using a multiexponential function in order to determine the effects of AK-C on the N- and L-current components. The (curve-fitted) N- and L-current components each declined ~ 50% during a 20 min recording in control

neurones. In the presence of 1 μg/ml AK-C the N- and L-components increased 5% and declined 15%, respectively, and in the presence of 50 μg/ml AK-C increased 50% and 15%, respectively. The inactivation time constant (τ_i) of the N-current component in control neurones, 150–200 ms, was reduced 25% in the presence of 50 μg/ml AK-C. The τ_i for the L-current component in control neurones, 1500–2500 ms, was unaffected by AK-C.

6. We concluded that AK-C had selective effects on the calcium components of nodose ganglion neurones. The N-current component was more sensitive to AK-C-dependent phosphorylation than the L-current component, and T-currents were unaffected. The decline in neuronal calcium currents during whole-cell recording may be due, at least in part, to a reduction in the intracellular concentration of AK-C, and the resultant dephosphorylation of calcium channels or associated proteins. Phosphorylation by AK-C may also result in the change of voltage-dependent properties of calcium channels.

INTRODUCTION

Calcium influx through voltage-sensitive channels influences diverse intraneuronal processes such as ion channel gating (Meech, 1978) and neurotransmitter release (Katz & Miledi, 1967). It is not surprising, therefore, that a variety of neurotransmitters and second messenger systems regulate calcium channel activity. In vertebrate cardiac myocytes, for example, ATP, cyclic AMP or the cyclic AMP-dependent protein kinase (AK) increase calcium currents or calcium channel activity (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; Cachelin, de Peyer, Kokubun & Reuter, 1983; Hartzell & Fischmeister, 1986; Belles, Malecot, Hescheler & Trautwein, 1988). In invertebrate neurones, elevation of intracellular cyclic AMP levels results in an increase in calcium currents (e.g. Hockberger & Connor, 1984), an effect presumably mediated by AK (Chad & Eckert, 1986; Eckert, Chad & Kalman, 1986). Armstrong & Eckert (1987) have shown that calcium channels in GH_3 cells, a clonal pituitary tumour cell line, require AK-induced phosphorylation to maintain normal activity.

These studies show that the cyclic AMP–AK system enhances or maintains voltage-sensitive calcium channel activity. Recent studies have shown, however, that multiple calcium channel types exist, the transient low-threshold (T), transient high-threshold (N) and slowly inactivating high-threshold (L) current components (Nowycky, Fox & Tsien, 1985; Gross & Macdonald, 1987; Wanke, Ferroni, Malgaroli, Ambrosini, Pozzan & Meldolesi, 1987; Fox, Nowycky & Tsien, 1987*a*, *b*). It is not yet certain which calcium current components are regulated by AK, as most of the above-mentioned studies were performed in cells that have primarily L-type currents, and N-type currents have been described primarily in vertebrate neurones or neural crest-derived cells (see also Docherty, 1988; Durroux, Gallo-Payet & Payet, 1988). We were interested, therefore, in determining if the cyclic AMP–AK system had differential effects on calcium current components in acutely dissociated vertebrate neurones.

In previous studies from this laboratory (Gross & Macdonald, 1988*a*, 1989*a*), we examined the effects of agents which increase intracellular cyclic AMP levels on the T-, N- and L-current components of cultured mouse dorsal root ganglion neurones.

The isolated T-current was unaffected by either forskolin or cyclic AMP, but currents containing the N- and L-current components were reduced by these agents. One limitation in interpreting those data (and those of some of the above-mentioned studies) was that agents which increase intracellular levels of cyclic AMP levels may affect calcium channel activity by means other than the activation of AK. For example, forskolin may have non-specific effects on other ion channels (Watanabe & Gola, 1987; Hoshi, Garber & Aldrich, 1988). We therefore performed these experiments, in which we examine the effect of the catalytic subunit AK (AK-C) on the calcium current components of nodose ganglion neurones. The results show that AK-C selectively affected calcium current components in a specific and concentration-dependent manner. Thus, AK-dependent phosphorylation may be an important mechanism for differential regulation of vertebrate neuronal calcium channel subtypes.

METHODS

Preparation of acutely dissociated neurones

Nodose ganglion neurones were prepared from 6- to 10-day-old rats, using a procedure modified from Ikeda, Schofield & Weight (1986) (see Gross, Wiley, Ryan-Jastrow & Macdonald, 1990). Briefly, rats were decapitated after ether anaesthesia; nodose ganglia were removed and treated with 1 mg/ml type Ia collagenase (Sigma Chemical Company, St Louis, MO, USA) in minimal essential medium (GIBCO Laboratories, Grand Island, NY, USA) buffered with 16·5 mM-$NaHCO_3$ for 25 min at 37 °C. The tissue was then transferred to a tube containing Dulbecco's minimum essential medium (GIBCO) and 5% fetal calf serum (GIBCO) to inhibit the enzyme, triturated gently to disperse the cells and then centrifuged and resuspended in minimum essential medium with Earle's salts supplemented with $NaHCO_3$ (16·5 mM), glucose (28·2 mM), nerve growth factor (10 ng/ml; Boehringer Mannheim, Indianapolis, IN, USA), penicillin (50 U/ml), streptomycin (50 mg/ml), $CaCl_2$ (3·6 mM) and 10% fetal calf serum (GIBCO). The suspension (approximately 0·5 ml/ganglion) was plated on 35 mm polystyrene Petri dishes (Corning Glass Works, Corning, NY, USA), without a substrate, 0·3 ml/dish and incubated at 37 °C in a 93% air, 7% CO_2 atmosphere. An additional 1 ml of medium was added after 30 min, and the cells were used for recordings within 1–12 h.

Preparation of solutions

Purified AK-C was prepared as described elsewhere (Olsen & Uhler, 1989) and stored at 4 °C for use within 24 h. Just before the experiment, the 1 mg/ml stock solution was diluted into the pipette recording solution (see below) at the stated concentrations. Experimental solutions were stored on ice; AK-C retained full activity for several hours (assayed as previously described; Uhler & McKnight, 1987).

For some experiments, the peptide inhibitor of AK (protein kinase inhibitor peptide, PKIP; Sigma) was dissolved in internal solution that contained 1 μg/ml ($\sim$ 20 nM) AK-C. An excess of PKIP was used, 0·2 mg/ml ($\sim$ 100 μM), to ensure inhibition of AK-C activity. The mixture was heated at 35 °C for 5–10 min. This solution was most effective immediately after heating, with a 25–50% loss of inhibition after 30–60 min, even if stored on ice. Reheating for 2–3 min was sufficient to restore the full inhibitory effect.

For all recordings in the presence of AK-C or AK-C-PKIP, the tip of the recording pipette was filled with standard recording solution, and the pipette was 'back-filled' with the experimental solution. Absence of protein-containing mixtures in the tip of the recording pipette facilitated the formation of gigaohm seals and helped prevent clogging of the pipette tip after patch rupture.

Whole-cell patch clamp recordings

Whole-cell voltage clamp recordings were obtained using the whole-cell variation of the patch clamp technique. Cells were bathed in a solution of the following (in mM): 67 choline chloride, 100 TEA, 5·6 glucose, 5·3 KCl, 5·0 $CaCl_2$, 10 HEPES (pH 7·3–7·4, 310–330 mosM; all reagents from Sigma). Glass recording patch pipettes (Fisherbrand microhaematocrit tubes, Pittsburgh, PA,

USA) with resistances of 0·5–1·5 MΩ were filled with recording solution consisting of the following (in mM): 140 CsCl, 10 HEPES, 10 EGTA, 5 ATP-Mg^{2+} and 0·1 GTP (all reagents from Sigma). The pH (7·3–7·4) was adjusted with 1 M-CsOH after the addition of ATP. The osmolality was 10–15% below that of the bath solution, 280–300 mosM.

Recordings were made at room temperature using the Axopatch 1-B patch clamp amplifier (Axon Instruments, Burlingame, CA, USA). Pipette and whole-cell capacitance and series resistance were corrected by compensation circuitry on the patch clamp amplifier. Typical initial input resistances were 500 MΩ to 1 GΩ. Series resistance was estimated by cancellation of the capacitance-charging current transient after patch rupture; typical values for the series resistance were 1·5–4 MΩ. In most cases, series resistance compensation of 80–90% was possible without significant noise or oscillation. Voltage step commands were generated, and currents were digitized (5 kHz), stored and analysed by a microcomputer (IBM AT or equivalent) using the program pCLAMP (Axon Instruments). The current traces were filtered with a Bessel filter at 10 kHz (−3 dB).

Analysis of current components

Leak current was estimated as the inverse of the current evoked using hyperpolarizing voltage commands of equal magnitude to the depolarizing commands used to evoke the inward currents. This current was digitally subtracted from the relevant inward current to obtain the calcium current.

The T-current was isolated by evoking currents from $V_h = -80$ or -90 mV at $V_c = -50$ to -20 mV; at more positive V_c more slowly inactivating current components were evoked (N and L). In order to distinguish between the N- and L-components qualitatively, we evoked currents at $V_c = +10$ or $+20$ mV, at 1 min intervals, alternating between V_h of -80 and -40 mV. We used the current evoked from $V_h = -40$ mV as an estimate of the L-current component, and the additional more rapidly inactivating current component evoked from $V_h = -80$ mV as an estimate of the N-current component.

We also used a multiexponential curve-fitting program (Asystant, Macmillan Software Company; see Gross & Macdonald, 1988*b*, 1989*a*) to determine quantitatively the effect of AK-C on the N- and L-current components (see also Bossu, Rodeau & Feltz, 1989). Current traces were truncated after the peak current (I_p) and before the voltage command offset, and then fitted using the following equation to perform a least-squares best fit:

$$y(t) = A_1\exp(-t/\tau_{11}) + A_2\exp(-t/\tau_{12}) + A_3\exp(-t/\tau_{13}) + A_4,$$

where $A_{1...3}$ represent the amplitudes of the current components, $\tau_{11...3}$ represent the corresponding inactivation time constants, and A_4 represents a fixed residual value (i.e. leak current). The majority of traces were better fitted (larger correlation coefficients and smaller residual errors) using the sum of two exponentials, as the T-current component was relatively small and truncated. Correlation coefficients for the fitted traces were routinely 0·99.

Statistical comparisons

Statistical comparisons were made using Student's two-tailed *t* test.

RESULTS

In the first series of experiments, we tested the effect of 50 μg/ml AK-C on the calcium current components of acutely dissociated rat nodose ganglion neurones. Currents were evoked in the absence and presence of 50 μg/ml AK-C at 1 min intervals, alternately from $V_h = -80$ mV and -40 mV at $V_c = +10$ mV (Fig. 1). In control neurones, calcium currents increased in magnitude during the first 5–7 min of the recording, and declined steadily thereafter (Fig. 1*Aa–c*). To allow comparison of currents, we normalized peak current magnitudes (I_p) to the maximal peak current, evoked from $V_h = -80$ mV, achieved in the first 5 min of whole-cell recording ($I_{p,max}$). Control currents, evoked from either V_h, declined 35–40% over 20 min.

In the presence of 50 μg/ml AK-C, currents were similar to controls in the first 5 min in all twenty-five neurones tested (Fig. 1*Ba–c*). For example, mean I_p values in one experiment for currents evoked from $V_h = -80$ mV were $2{\cdot}81 \pm 0{\cdot}3$ nA in control neurones (mean ± S.E.M. $n = 7$) and $2{\cdot}58 \pm 0{\cdot}3$ nA in the presence of AK-C

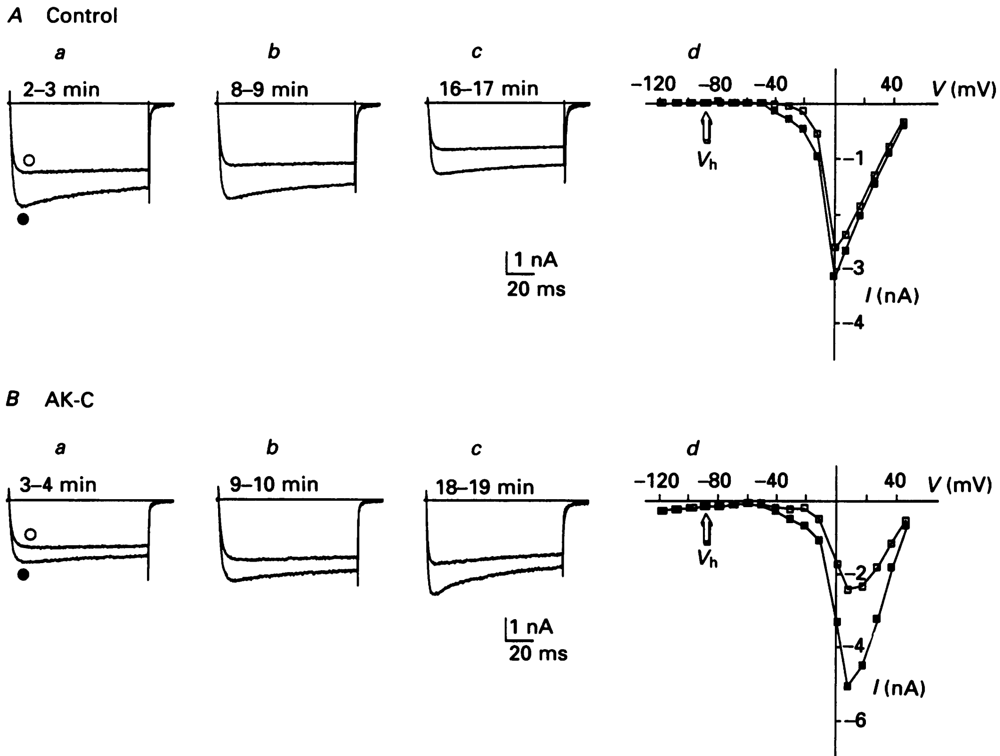
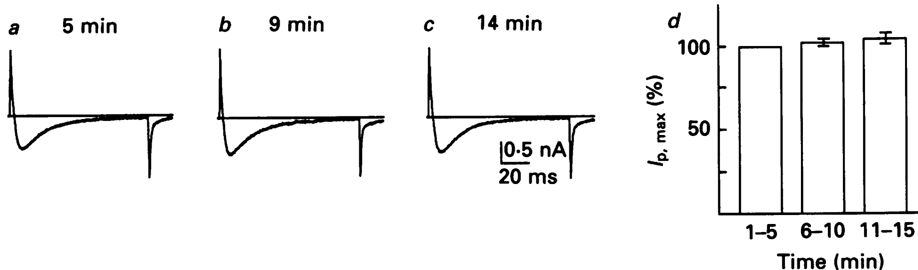


Fig. 1. AK-C increased the N- and L-current components in acutely dissociated rat nodose ganglion neurones. In all figures, currents are leak subtracted and inward currents are downward. In this figure, calcium currents from two different neurones are shown. Currents were evoked at $V_c = +10$ mV from $V_h = -80$ mV (●) and $V_h = -40$ mV (○) in the absence (*Aa–c*) or presence of 50 μg/ml AK-C, included in the recording pipette (*Ba–c*); the traces were recorded at the times indicated. Current–voltage relations were derived from currents evoked from $V_h = -90$ mV at V_c ranging from -120 to $+50$ mV in the same neurones 22 min after patch rupture (*Ad*, *Bd*). Peak current (I_p; ■) and late current (I_{100}; □) values are plotted.

($n = 13$). Thereafter, currents recorded in the presence of AK-C increased in magnitude; currents evoked from $V_h = -80$ mV increased 30–35% over 20 min, and currents evoked from $V_h = -40$ mV increased 10–15%. Unlike control currents, in which the rapidly inactivating current component was progressively lost, this component was increased in currents recorded in the presence of AK-C, particularly in currents evoked from $V_h = -80$ mV.

We derived current–voltage plots from currents recorded in the absence (Fig. 1*Ad*) and presence (Fig. 1*Bd*) of 50 μg/ml AK-C. Neurones were held at a $V_h = -90$ mV,

and hyperpolarizing and depolarizing voltage commands were applied every 10 s, with V_c ranging from -120 mV to $+50$ mV. T-current was evoked at V_c at or positive to -50 mV in both the absence and presence of AK-C. In control neurones, the maximal I_p was achieved at V_c between -10 and $+10$ mV, but in the presence of AK-



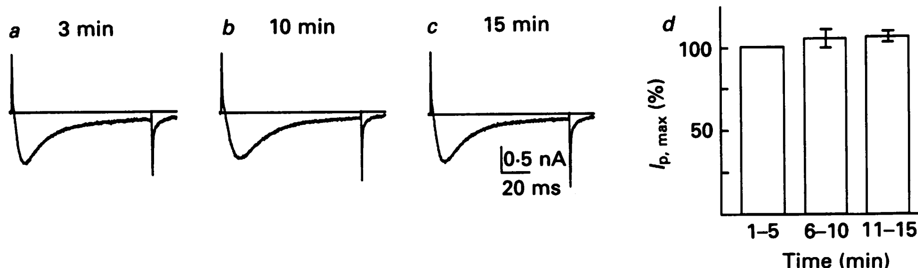


Fig. 2. T-currents were unaffected by AK-C. T-currents were evoked at $V_c = -20$ mV from $V_h = -80$ mV using 100 ms commands in the absence (*Aa–c*) or presence (*Ba–c*) of 50 μg/ml AK-C. Traces from two neurones are shown. Mean peak current (I_p) values ($\pm$S.E.M.) are plotted as a function of time after patch rupture (*Ad* and *Bd*; $n = 6$ neurones for each group).

C, this range was shifted to more positive potentials, between 0 and $+30$ mV. In fourteen control neurones, for example, the maximal I_p occurred at a mean V_c of $-1·5 \pm 1·8$ mV, compared to $+13·3 \pm 1·7$ mV in the presence of AK-C ($n = 21$ neurones). We also plotted the current–voltage relation of the late current (I_{100}) as a means of showing the extent of inactivation occurring at each V_c. At 22 min after patch rupture, there was little difference between I_p and I_{100} in control neurones, whereas in AK-C neurones, a large difference was evident. The amount of inactivation seen in the presence of AK-C late in the recording was similar to or greater than that seen in control neurones early in the recording (data not shown).

The current–voltage plots suggested that AK-C had little or no effect on T-current. To test this directly, we recorded isolated T-currents in the absence and presence of 50 μg/ml AK-C (Fig. 2). Currents were evoked from $V_h = -80$ mV at $V_c = -65$ to -10 mV at 1–2 min intervals after patch rupture. In control neurones, T-currents did not change significantly during the recording period, unlike the current

components evoked at more positive V_c (Fig. 2*Aa–d*). T-currents recorded in the presence of AK-C had similar activation and inactivation rates, and current magnitudes were similar to controls ($0{\cdot}63 \pm 0{\cdot}11$ nA, compared to controls, $0{\cdot}79 \pm 0{\cdot}17$ nA; mean ± S.E.M. $n = 6$ neurones in each group). There was no change in current magnitude over time in the presence of AK-C (Fig. 2*Ba–d*).

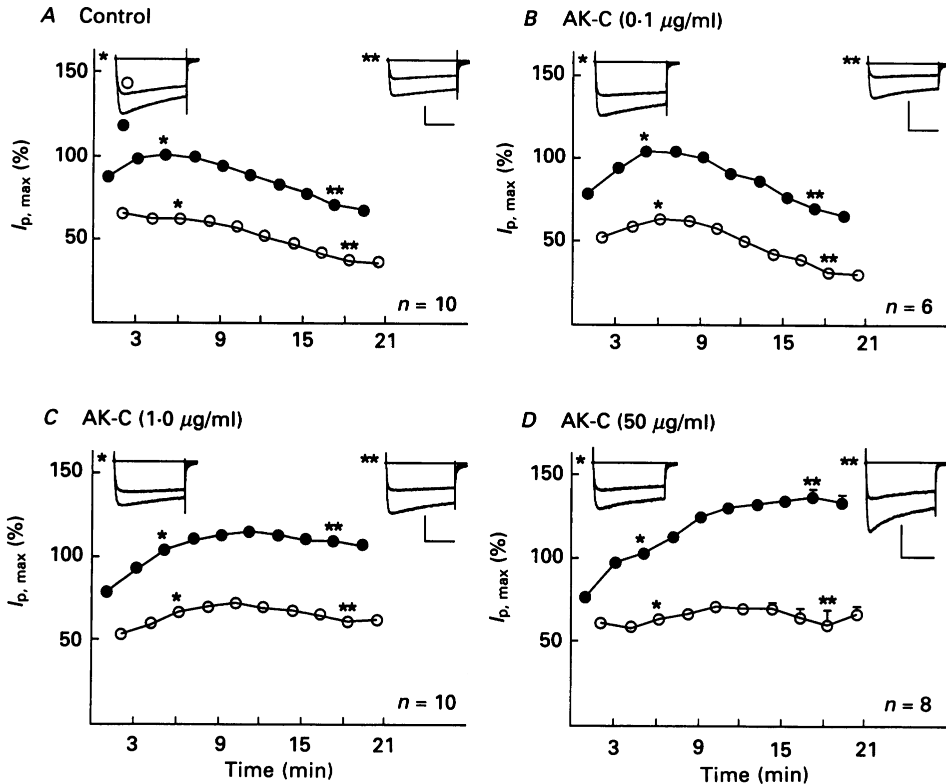


Fig. 3. The effect of AK-C was concentration dependent. Currents were evoked at $V_c = +10$ mV from $V_h = -80$ (●) or -40 mV (○) in the absence (*A*) or presence of 0·1, 1 or 50 μg/ml AK-C (*B–D*, respectively). Representative current traces from four different neurones are shown, recorded 5–6 (*) and 17–18 (**) min after patch rupture. Pooled data are shown below the traces, with the sample number indicated; error bars are shown in one direction only, unless they fall within the symbol. The symbols represent the mean I_p value expressed as a percentage of $I_{p,max}$, i.e. the maximal value of I_p evoked from $V_h = -80$ mV within the first 5 min of the recording. Calibration bars indicate 2 nA (vertical) and 40 ms (horizontal).

The above experiments showed that AK-C had selective effects on the calcium current components of nodose ganglion neurones. In order to determine if the observed effects of AK-C on calcium current were specific, we performed two different experiments. In the first, we tested the effect of different concentrations of AK-C, ranging from 0·1 to 50 μg/ml, and in the second, we tested the ability of PKIP to reverse the effects of AK-C on calcium currents.

Calcium currents were evoked at 1 min intervals after patch rupture from $V_h = -80$ mV and -40 mV in the presence of 0·1, 1 and 50 μg/ml AK-C (Fig. 3). Currents recorded in the presence of 0·1 μg/ml AK-C were similar to those in control neurones, the rate of run-down being virtually identical (compare Fig. 3*A* and *B*).

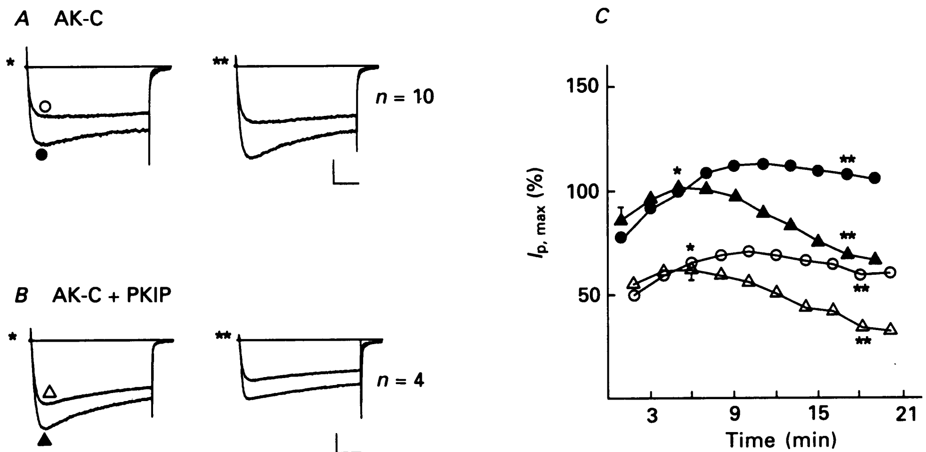


Fig. 4. The effect of AK-C was reversed by a specific peptide inhibitor (PKIP). Currents were evoked at $V_c = +10$ mV from $V_h = -80$ (●, ▲) and -40 mV (○, △) in the presence of 1 μg/ml AK-C and in the absence (*A*; ○, ●) or presence of 0·2 mg/ml PKIP (*B*; △, ▲). The illustrative currents were recorded at 5–6 (*) and 17–18 (**) min after patch rupture. Pooled data from the indicated number of neurones are shown to the right of the traces; the symbols represent the mean I_p values of the currents, expressed as a percentage of $I_{p, max}$. The error bars are shown in one direction only unless they fall within the symbol. The calibration bars represent 1 nA (vertical) and 20 ms (horizontal).

Currents recorded in the presence of 1 μg/ml AK-C were similar to control currents initially (Fig. 3*C*). Thereafter, currents evoked from $V_h = -80$ mV increased to 110 ± 5% of $I_{p, max}$ by 11–12 min ($n = 10$ neurones; $P < 0.05$ compared to control) and currents evoked from $V_h = -40$ mV increased from 50 ± 4 to 66 ± 5% of $I_{p, max}$ by 11–12 min ($P < 0.05$ compared to control). These currents declined slightly over the next 5–10 min. Currents recorded in the presence of 50 μg/ml AK-C, evoked from $V_h = -80$ mV, increased to 133 ± 8% of $I_{p, max}$ at 17–18 min ($n = 8$ neurones; $P < 0.01$ compared to control). In most neurones, this current was maintained for an additional 10–20 min. The current evoked from $V_h = -40$ mV increased from 57 ± 7 to 66 ± 7% of $I_{p, max}$ by 11–12 min ($P < 0.05$ compared to control), with a slight decline thereafter (Fig. 3*D*).

This experiment showed that the effect of AK-C was concentration and time dependent and was greater on currents evoked from $V_h = -80$ mV than on currents evoked from -40 mV, independent of the concentration of AK-C. To determine the specificity of these effects, currents were recorded in the presence of AK-C and a specific peptide inhibitor of AK-C, PKIP. In this experiment, the recording pipette contained AK-C (1 μg/ml) with or without PKIP (0·2 mg/ml; Fig. 4). PKIP completely blocked the effect of AK-C on calcium currents (Fig. 4*A* and *B*). Current magnitudes and rates of run-down were identical to those seen in controls (compare

Fig. 3*A* and *C* with Fig. 4). We also recorded calcium currents in the presence of 50 μg/ml AK-C that had been boiled for ⩾ 10 min. Boiling reduced the activity of AK-C 90–95 %, reduced the magnitude of the effect on calcium currents to that seen with 1 μg/ml AK-C and delayed the onset of the effect, which was still evident within

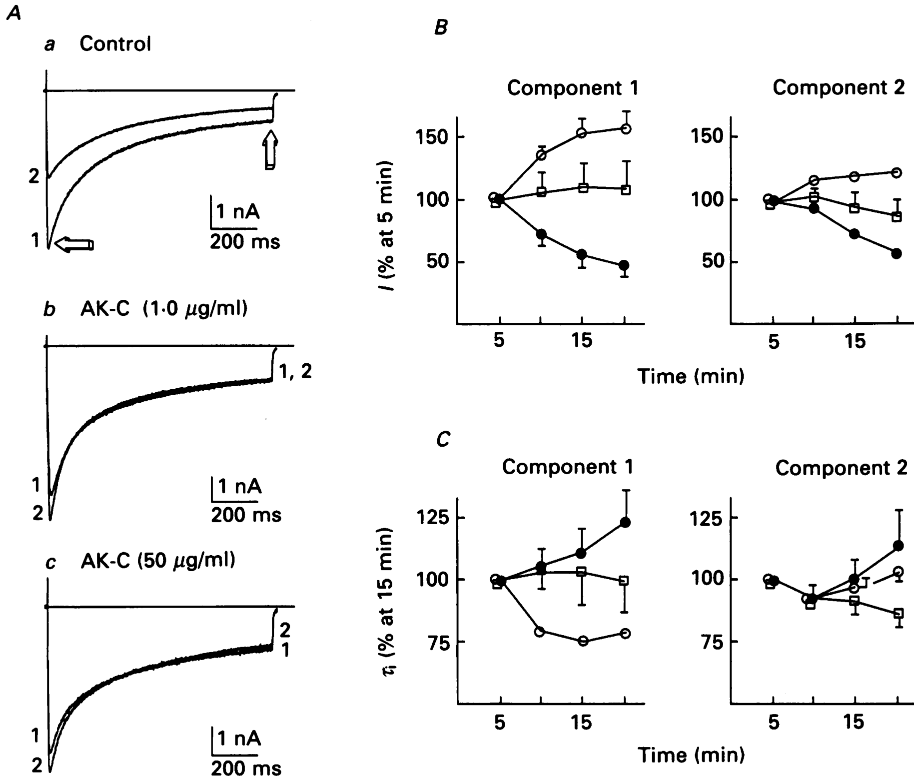


Fig. 5. AK-C increased both the N- and L-current components and reduced the τ_i of the N-current component. Currents were evoked at $V_c = +10$ mV from $V_h = -80$ mV using 1000 ms voltage commands at 5 min intervals in the absence (*Aa*) and presence of 1 μg/ml (*Ab*) or 50 μg/ml AK-C (*Ac*). The illustrative current traces were recorded at 5 min (1) and 15 min (2) after patch rupture. Traces were truncated (arrows) and fitted with a multiexponential function (see Methods). Curve-fitted amplitudes (*B*) and τ_i (*C*) for the N-current component (component 1) and L-current component (component 2) are shown as a function of time after patch rupture for currents recorded in the absence (●, $n = 9$) and presence of 1 μg/ml AK-C (□, $n = 4$) or 50 μg/ml AK-C (○, $n = 10$). The symbols show the mean values of the number of samples indicated; error bars are shown in one direction only unless they fall within the symbol.

20 min (data not shown). In addition, we recorded calcium currents with 50 μg/ml bovine serum albumin in the recording pipette, and found that the currents were similar to controls (data not shown).

We next evoked calcium currents using long (1 s) voltage commands and used curve-fitting analysis to determine the τ_i and relative proportions of the N- and L-current components. Currents were evoked at $V_c = +10$ mV from $V_h = -80$ mV at 5 min intervals after patch rupture in the absence and presence of 1 and 50 μg/ml

AK-C. In this way, we were able to observe time-dependent changes occurring in either the amplitude of the current components or in their inactivation rates (Fig. 5). In control neurones, calcium currents declined at a rate similar to that observed in other experiments (Fig. 5*Aa*). Currents recorded in the presence of both 1 and 50 μg/ml AK-C showed an increase in the I_p during the recording, with an associated increase in the rate of current inactivation. The late current (I_{1000}) changed little during the recording in the presence of 1 μg/ml AK-C, but in most neurones was slightly smaller in the presence of 50 μg/ml AK-C (Fig. 5*Ab* and *c*).

The effect of AK-C on the curve-fitted current components was concentration dependent. In control neurones, the faster inactivating current component (component 1 or N-current component) decreased 55 ± 9% during the recording, compared to its initial value at 5 min (Fig. 5*B*, ●, $n = 9$). In contrast, the N-current component recorded in the presence of 1 μg/ml AK-C increased 5 ± 22% (Fig. 5*B*, □, $n = 4$; $P < 0{\cdot}05$ compared to control) and, in the presence of 50 μg/ml AK-C, increased 53 ± 13% by 20 min (Fig. 5*B*, ○, $n = 10$; $P < 0{\cdot}001$ compared to control).

There was a similar concentration-dependent effect on the longer inactivating current component (component 2 or L-current component). The control L-current component remained stable during the initial recording period (up to 10 min), and then declined 46 ± 5% by 20 min. In the presence of 1 μg/ml AK-C, the L-current component decreased 18 ± 14% by 20 min ($P < 0{\cdot}05$ compared to control) and, in the presence of 50 μg/ml AK-C, increased 16 ± 2% by 20 min ($P < 0{\cdot}01$ compared to control).

The N-current component had initial τ_i ranging from 130 to 392 ms, with a mean value of 195 ± 28 ($n = 9$). Because of this wide range, we plotted the τ_i as a percentage of the initial τ_i for each neurone (Fig. 5*C*). The τ_i of the N-current component increased slightly during the recording in control neurones, although the difference did not reach statistical significance. In the presence of 1 μg/ml AK-C, the τ_i of the N-current component remained the same throughout and, in the presence of 50 μg/ml AK-C, was reduced 23 ± 4% ($P < 0{\cdot}01$ compared to control).

Unlike the N-current component, there were no statistically or concentration-dependent changes in the τ_i of the L-current component. Initial τ_i ranged from 1150 to 3100 ms, with a mean value of 1827 ± 210 ms.

DISCUSSION

The present experiments provide a direct demonstration that AK-C had a specific and selective action on the calcium current components of rat nodose ganglion neurones. This effect was present at physiological concentrations of AK-C (50 μg/ml, about 1 μM; Hofmann, Bechtel & Krebs, 1977), was concentration dependent and was reversed by a specific peptide inhibitor of AK-C. AK-C had no effect on the T-current component, and increased the N- and L-current components; furthermore, our data support the idea that the N-current component was more affected than the L-current component by AK-C. This was particularly evident when comparing currents evoked from different V_h. AK-C had a greater effect on currents evoked from $V_h = -80$ mV, which contained a greater proportion of the N-current component than currents evoked from $V_h = -40$ mV, which consisted primarily of the L-current

component. The curve-fitting analysis also supported this interpretation. We conclude that AK-mediated phosphorylation of N and L, but not T, calcium channels or associated proteins is required for full channel activity. The present results suggest that a major effect of phosphorylation is to increase the number of channels available for opening (See Armstrong & Eckert, 1987) or, less likely in our opinion, to increase single-channel conductance. We favour the view that calcium channels are themselves the target for AK-C-dependent phosphorylation (Nunoki, Florio & Catterall, 1989) rather than associated proteins such as G proteins, which do not appear to be substrates for AK (Premont & Iyengar, 1989).

AK-C had a greater effect on the current components that 'ran down' during whole-cell recording conditions; this suggests that the loss of AK may be responsible, in part, for the run-down of N- and L-, but not T-current components. This confirms other work, that cyclic AMP or AK prevented current run-down (see Introduction), but extends this observation to vertebrate neurones which have multiple calcium current components. In fact, the rate and extent of N- and L-current loss was dependent on intracellular 'dialysis', as run-down was faster with lower resistance electrodes and the extent of current loss was independent of stimulus frequency under our recording conditions (Gross, R. A. & Macdonald, R. L., unpublished observations). In support of this idea is the recent observation that inclusion of tissue extract in recording pipettes prevents run-down in cardiac cells (Kameyama, Kameyama, Nakayama & Kaibara, 1988).

There were, in addition, changes in voltage-dependent characteristics of currents evoked in the presence of AK-C. The N- and L-current components were evoked at slightly more positive V_c in the presence of AK-C than in control neurones. We consider it unlikely that this was a non-specific effect of AK-C because a similar shift was not observed for the T-current component. The shift in the current–voltage relation in the presence of AK-C could be due to an increase in the L-current component, which activates at more positive potentials (Fox *et al.* 1987*a*, *b*), to a change in the voltage dependency of current activation of either the N- or L-component or to an uncompensated series resistance error secondary to increased calcium conductance. Regarding the last possibility, we have calculated that a 30% increase in conductance under recording conditions would produce a voltage error of 2–4 mV, smaller than the shift observed, but our data do not allow us to determine the actual error. Currents also inactivated faster in the presence of AK-C. This could be due to an increase in the N-current component or to a change in channel inactivation, or both. It is also possible that faster current inactivation was the result of an increase in calcium influx and calcium-dependent inactivation (Chad & Eckert, 1986), although, in some neurones, currents inactivated faster before increases in I_p were observed. Our results are therefore consistent with the idea that AK-mediated phosphorylation may change voltage-dependent properties of calcium channels, but further experiments will be required to determine the identity of affected channels and the mechanisms by which AK-C regulates channel activity.

We performed these experiments with the catalytic subunit of AK in order to determine its effects on calcium currents directly. In a previous study from this laboratory, using the single-electrode voltage clamp technique in cultured dorsal root ganglion neurones, forskolin had effects similar to those of AK-C; the L-current

component was increased slightly and the inactivation rate of the N-current component was increased (Gross & Macdonald, 1989*a*). Forskolin also reduced the N-current component, a finding not observed in the present study. To resolve this discrepancy, we have applied forskolin (100 μM) to nodose neurones during whole-cell recording. The results of these preliminary experiments were similar to those previously reported; in particular, forskolin did not produce an increase in current, and reduced currents evoked from V_h positive to -80 mV (Gross, R. A. and Macdonald, R. L., unpublished observations). It is possible that the effects of forskolin were obscured by intracellular dialysis and removal of either cyclic AMP or AK from the cytoplasm, but it may be that the reduction of calcium current by forskolin was an effect unrelated to the increase in intracellular cyclic AMP levels or an increase in AK activity (cf. Watanabe & Gola, 1987; Hoshi *et al.* 1988). In any event, the difference in the effects of forskolin and AK-C in our studies underscore the importance of comparing the effects of agents which activate AK indirectly with the direct actions of AK-C.

The regulation of neuronal voltage-sensitive calcium channel activity in vertebrate neurones by AK could result in marked changes in calcium-dependent processes such as neurotransmitter release. A variety of neurotransmitters affect neuronal calcium currents via GTP-binding proteins (e.g. Dunlap & Fischbach, 1981; Dolphin, Forda & Scott, 1986; Macdonald, Skerritt & Werz, 1986; Gross & Macdonald, 1987; Wanke *et al.* 1987; Walker, Ewald, Perney & Miller, 1988), and some may do so via the adenylate cyclase–cyclic AMP system (e.g. Byrne, 1985; Bhoola & Pay, 1986; Larocca, Ledeen, Dvorkin & Makman, 1987). It is of interest in this regard that another G protein-linked second messenger system, the phosphatidylinositol C kinase system, also regulates vertebrate neuronal calcium currents (Rane & Dunlap, 1986; Gross & Macdonald, 1989*b*). Thus, the final state of neuronal calcium channels may be dependent on the full complement of neurotransmitter receptors and associated G proteins as well as on the activity of second messenger systems such as the cyclic AMP–AK system.

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