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# Blockade of Ca-activated K conductance by apamin in rat sympathetic neurones

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**1** Effects of apamin on rat sympathetic neurones were investigated by means of intracellular and extracellular recording.

**2** Apamin (50 nM) significantly shortened the after-hyperpolarization (AH) following the spike evoked by current injection and slightly decreased its peak amplitude without affecting the time course of the spike.

**3** The AH following the synaptically-evoked spike was also blocked by apamin. This effect was dose- and time-dependent ($ID_{50}$ estimated by extracellular recording approximately 15 nM, 20 min after application) and poorly reversible. Transmission of a single volley was not affected by 50 nM apamin.

**4** Though a long depolarizing current caused one or two spikes in the cell, greater repetitive firing was observed in the presence of apamin. Spontaneous repetitive firing, however, was not observed except for anodal-break spikes. Resting potential and input membrane resistance were essentially unchanged by apamin.

**5** The maximum rate of rise of the Ca spike was not decreased by 50 nM apamin but the duration of the spike was lengthened by 60%. The AH following the Ca spike was also blocked by apamin.

**6** These results suggest that apamin suppressed the slow AH without any inhibition of the Ca flux into the cell and is useful as a blocker of $G_{K(Ca)}$ in the rat sympathetic neurone.

## Introduction

A Ca-activated K channel has been found in various excitable and non-excitable cells (see Schwarz & Passow, 1983; Petersen & Maruyama, 1984), including the rat sympathetic neurone (Brown *et al.*, 1982; Galvan & Sedlmeir, 1983), and may have various physiological roles. In sympathetic neurones, $G_{K(Ca)}$ may generate a prolonged spike after-hyperpolarization (AH) (McAfee & Yarowsky, 1979; Brown *et al.*, 1982), regulate repetitive firing (Kuba *et al.*, 1983), and accelerate spike repolarization (MacDermott & Weight, 1982; Brown *et al.*, 1983; Galvan & Sedlmeir, 1984).

Apamin is a polypeptide from bee venom. K-flux studies have suggested that apamin blocks the Ca-activated K channels in rat brain (Seagar *et al.*, 1984), guinea-pig intestinal smooth muscle (Banks *et al.*, 1979; Maas *et al.*, 1980) and guinea-pig hepatocytes (Burgess *et al.*, 1981; Cook & Haylett, 1985). Specific binding of $^{125}$I-apamin has been shown in brain synaptosomes (Hugues *et al.*, 1982a), neuroblastoma cells (Hugues *et al.*, 1982b), skeletal muscle in culture (Hugues *et al.*, 1982c), colon (Hugues *et al.*, 1982d) and hepatocytes (Cook & Haylett, 1985). A large difference among species and tissues has been observed (Habermann & Fischer, 1979; Cook & Haylett, 1985).

In electrophysiological studies, it has been demonstrated that apamin blocks AH or $I_{K(Ca)}$ (neuroblastoma cells, Hugues *et al.*, 1982b; skeletal muscle cells, Hugues *et al.*, 1982c). Apamin is, however, rarely used as a blocker of $G_{K(Ca)}$, since it is not effective in the molluscan neurones (Hermann & Hartung, 1983). Although it was originally reported as also ineffective in bullfrog sympathetic ganglion cells (Brown *et al.*, 1983), recently Pennefather *et al.* (1985) have shown two distinct components of $I_{K(Ca)}$ and the slow component is apamin-sensitive. Lack of a specific and direct blocker has hindered investigation of the physiological functions of $G_{K(Ca)}$ (Brown *et al.*, 1983).

We have previously shown that crude bee venom suppressed the AH in the rat sympathetic ganglion (Kawai *et al.*, 1985). In the present study, we inves-

tigated the effects of purified apamin and demonstrated its potent and direct blockade of the Ca-activated K channel.

## Methods

Superior cervical ganglia were excised from male Wistar rats weighing 300–450 g. The isolated ganglia were immersed in physiological saline and the connective tissue sheath was carefully removed under a binocular microscope. Thereafter, the preparation was placed in an organ bath and superfused with saline.

The physiological saline had the following composition (mM): NaCl 137.9, KCl 4.0, $CaCl_2$ 2.0, $MgCl_2$ 0.5, $NaHCO_3$ 12.0, $KH_2PO_4$ 1.0, glucose 11.1. The solution was gassed with 5% $CO_2$ and 95% $O_2$. All experiments were carried out at pH 7.4 and at about 34°C. In experiments on the $Ca^{2+}$ spike, additional $CaCl_2$ and tetraethylammonium chloride were substituted for equimolar amounts of NaCl.

Intracellular recordings were made with glass microelectrodes containing 3M KCl and having a resistance between 35 and 60 Mohm. Current was applied through the recording elelctrode by use of an active bridge circuit. Action potentials were evoked by anodal current of 2–5 ms (for $Na^+$ spike) or 10 ms (for $Ca^{2+}$ spike) duration across the cell membrane. Synaptically-evoked action potentials were intracellularly and/or extracellularly recorded. The pre-ganglionic nerve was drawn into a suction electrode and stimulated with 0.2 ms rectangular pulses. For extracellular recording, the internal carotid nerve was drawn into another suction electrode and the potentials were amplified through a.c. coupling of 1 s time constant. The potentials were displayed on an oscilloscope (model VC-9, Nihon-Kohden Ltd.) and permanently recorded on oscillograph paper and, occasionally, the synaptic potentials were continuously recorded on a pen recorder (model FBR-252A, TOA Electronics Ltd.) using a peak height detector modified from that of Courtice (1977).

In experiments with microelectrodes, collection of data was started 30 min after an impalement in order to make sure of stable recording; recorded potentials did not deteriorate significantly over further periods.

The drugs used were: hexamethonium chloride dihydrate, tetraethylammonium chloride (from Wako Pure Chemicals), tetrodotoxin (gift from Prof. Okuda, Tokyo Univ.), apamin (control: E, from Serva).

## Results

The directly-evoked action potential was followed by a prolonged after-hyperpolarization (AH) which had two components. One component was a fast negative peak and the other a slowly decaying component; these components overlapped each other in many cells (see Figure 1). It has been established that the prolonged AH is a result of activation of Ca-activated K channels (McAfee & Yarowsky, 1979).

Apamin at 50 nM markedly shortened the AH following a directly-evoked action potential and suppressed its slowly decaying component (Figure 1). The mean half decay time of the AH was 86.5 ± 29.1 ms ($n = 10$) in control and 7.1 ± 1.4 ms ($n = 10$) in the presence of 50 nM apamin. This effect developed progressively and reached a maximum within 5 min. At 15 nM it took about 10 min to achieve a maximum effect of the same magnitude. When stimulation was started 5 min after application of 50 nM apamin, no progressive increase in the magnitude of inhibition was observed. This observation rules out the possibility that the blockade was use-dependent. The recovery from the depression was very slow and full recovery was not observed even after 1 h. When fresh cells were impaled after preincubation in 50 nM apamin for 10 min or more, no cells with a prolonged AH were observed, indicating that the suppression of AH after apamin was not due to a deteriorating impalement.

The peak of the AH (16.4 ± 1.0 mV in control, $n = 10$) was decreased in amplitude to 12.7 ± 0.8 mV ($n = 10$) in the presence of apamin, but the parameters for the configuration of the spike potential, such as amplitude (69.0 ± 2.3 mV in control, $n = 10$), duration at half amplitude (1.5 ± 0.1 ms), maximum rate of rise (66.8 ± 5.6 $Vs^{-1}$) and maximum rate of fall (31.7 ± 2.9 $Vs^{-1}$), were not changed by apamin (Table 1).

Figure 2 shows the AHs following the orthodromic action potential. The time course of the AH was varied by the intensity of pre-ganglionic nerve stimulation. When the intensity was just threshold for the spike, the AH had fast and slow phases, while at supramaximal intensity only the slow phase was observed. Apamin (50 nM) significantly suppressed the slow phase of the AH, the fast phase remaining as with direct stimulation. When the pre-ganglionic nerve was repetitively stimulated with supramaximal intensity, each pulse never failed to evoke a spike. On submaximal stimulation (just threshold for first spike), only e.p.s.ps were usually observed during the AH, but these e.p.s.ps could develop spikes after an application of 50 nM apamin (Figure 2A).

Since the slow component was almost completely suppressed by apamin on pre-ganglionic stimulation, the time-course and dose-dependency of the effect of apamin was quantitatively examined. Extracellular recording was used to measure the averaged response of many cells over a long period. Recorded responses following supramaximal pre-ganglionic stimulation

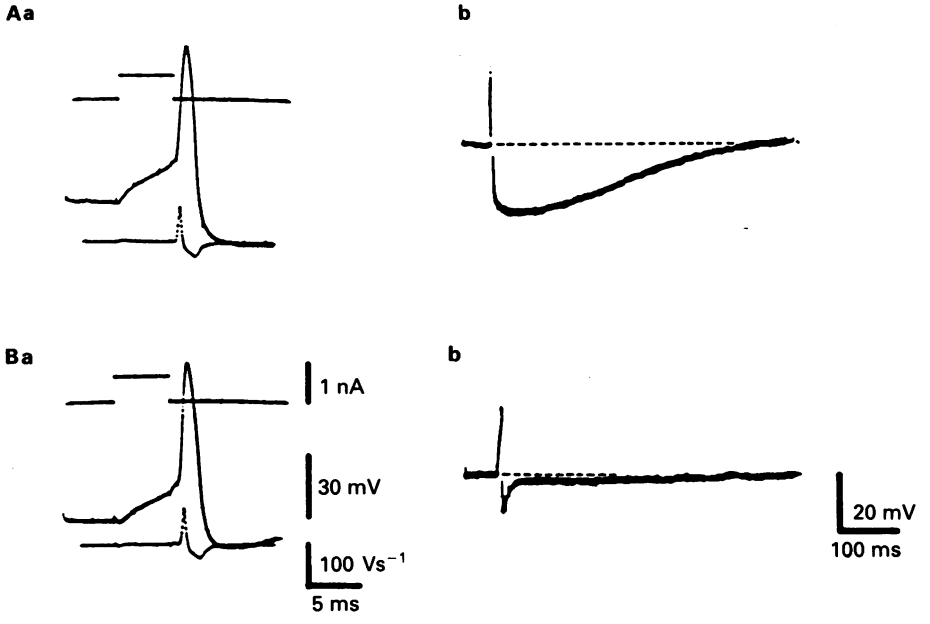


**Figure 1** Effects of apamin on directly-evoked action potentials (a) and after hyperpolarization (AH) (b). In (a) traces depict from top to bottom, applied currents, action potentials and their first derivatives, respectively. (A) control; (B) 3 min after apamin (50 nM).

consisted of a compound action potential followed by a slow hyperpolarization. The time course of the latter was consistent with that of the AH simultaneously recorded intracellularly from a superficial cell (Figure 3A). The inhibitory effects of apamin on the slow hyperpolarization were time- and dose-dependent. A residual component in the presence of 50 nM apamin was not affected by raising the concentration to 150 nM. An apamin-resistant and slowly declining small component was similarly observed using intracellular recording (Figure 1). Though further examination was not done because of its small amplitude, there is a possibility of the involvement of an apamin-insensitive K-conductance or of an electrogenic pump.

The amplitude of the compound action potential evoked at a frequency of 0.1 Hz was not influenced by 50 nM apamin (amplitude in the presence of apamin 99.4 ± 1.0% ($n$ = 14) of control). When the amplitude was reduced to about 30% by pretreatment with 0.3 mM hexamethonium, apamin still did not show any significant effects (96.0 ± 2.3%, $n$ = 4).

In intracellular experiments we examined the effects of apamin on the accommodation of the membrane to depolarizing current and other membrane properties. When a long depolarizing current was injected through the intracellular electrode into the cell (up to 1 nA), one or two action potentials usually occurred (Figure 4A). In the presence of apamin (50 nM), the accommodation of the cell membrane was significantly decreased and the repetitive firing of spikes was observed, frequency of firing depending upon current intensity. Apamin (50 nM) did not affect the resting membrane potential (− 54 ± 1 mV in control, $n$ = 10). The input membrane resistance (41.7 ± 4.2 Mohm in control, $n$ = 9) was essentially not changed by 50 nM apamin (111.0 ± 5.6% of control, $n$ = 9). The hyperpolarizing electrotonic potiential slowly declined from its peak (Figure 4B), probably due to a time-dependent rectification by M-current (Adams *et al.*, 1982). Lack of change in the shape of electrotonic potential may indicate that apamin did not affect M-current. In the presence of apamin the probability of occurrence of an anodal-break spike was not affected, but the spike was occasionally accompanied by a second spike (Figure 4B). No other increase in spontaneous activity was noted. The I-V relationship of the cell membrane was not affected by apamin (Figure 4C).

A Ca-dependent action potential (Ca spike) could be evoked in the presence of 1 μM tetrodotoxin, 10 mM tetraethylammonium and 4 mM extracellular $Ca^{2+}$ (Figure 5). The amplitude, duration (at half amplitude), maximum rate of rise and maximum rate of fall of Ca spike were 52.1 ± 2.2 mV, 15.9 ± 1.8 ms, 7.0 ± 1.5 $Vs^{-1}$ and 6.1 ± 1.2 $Vs^{-1}$, respectively ($n$ = 7). The AH had a peak amplitude of 15.5 ± 1.0 mV and half-decay time of 143.0 ± 16.3 ms ($n$ = 7). Apamin (50 nM) markedly depressed the AH and prolonged the duration of the Ca spike without decreasing the

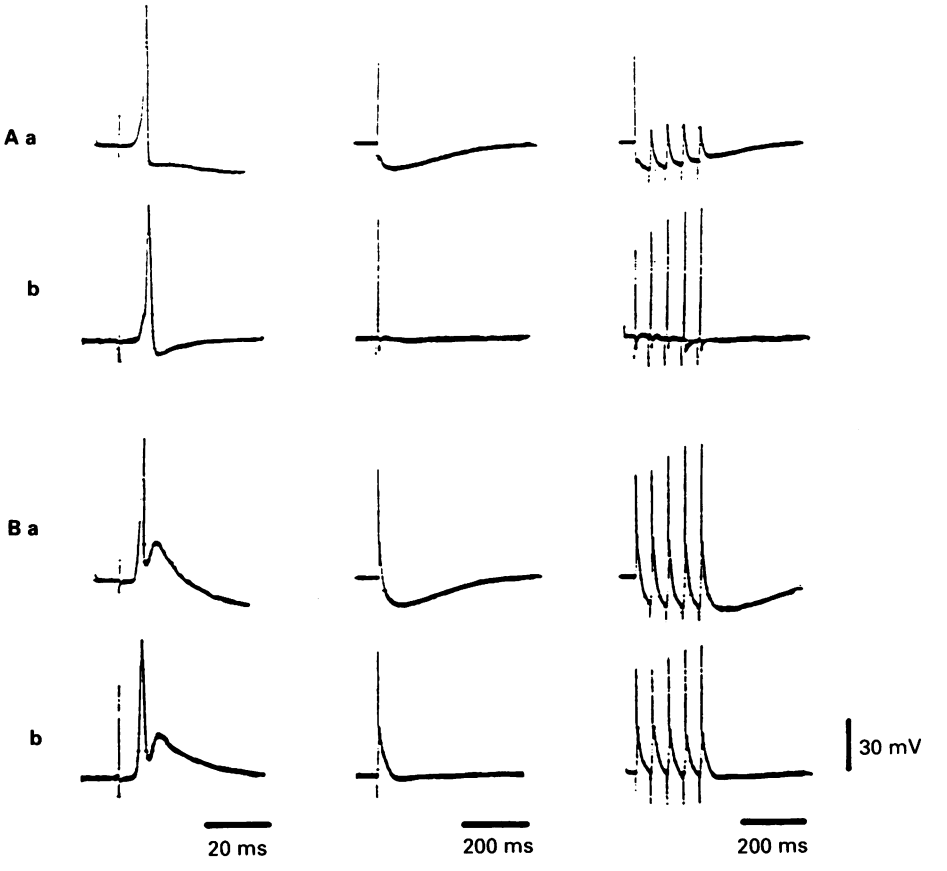


**Figure 2** Effects of apamin on the synaptically-evoked action potentials and AH. Preganglionic nerve stimulation (0.2 ms in duration) was submaximal at just threshold for the spike (A) and supramaximal voltage (B). (Aa) and (Ba) control; (Ab) and (Bb) 5 min after apamin (50 nM). In the right hand panels, 5 pulses were applied to the preganglionic nerve at 20 Hz. In the slow traces, action potential peaks are not shown.

rising phase (Figure 5, Table 1). As with the Na spike, repetitive spikes were observed during a prolonged injection of depolarizing current.

**Table 1** Effects of apamin (50 nM) on parameters of the action potential and after hyperpolarization relative to controls in rat sympathetic neurones

| | *Na spike* | *Ca spike* |
|---|---|---|
| Spike | | |
| Amplitude | 99.8 ± 0.5% | 105.5 ± 2.1% |
| Duration | 100.7 ± 0.5% | 162.8 ± 11.9% |
| Max. rate of rise | 99.8 ± 2.1% | 113.0 ± 3.4% |
| Max. rate of fall | 100.0 ± 1.9% | 72.1 ± 6.7% |
| After-hyperpolarization | | |
| Peak amplitude | 79.7 ± 6.0% | 68.6 ± 4.0% |
| Half decay time | 10.7 ± 2.7% | 26.0 ± 6.2% |
| | $n = 10$ | $n = 7$ |

## Discussion

The present study demonstrates that apamin is a blocker of the Ca-activated K conductance ($G_{K(Ca)}$) in rat sympathetic neurones. It is well established that the prolonged AH is due to an activation of $G_{K(Ca)}$. This results from an increase in cytoplasmic free Ca (Smith *et al.*, 1983; Kuba *et al.*, 1983) due to Ca influx through voltage-dependent Ca channels during the action potential. Apamin selectively inhibited the AH following Na or Ca spikes without any depression of the maximum rate of rise of the Ca spike. These observations suggest that apamin does not inhibit the Ca flux into the cells but directly blocked the $G_{K(Ca)}$ in rat sympathetic neurones.

Kinetic studies using voltage clamp technique suggest that the repolarization from the peak of the action potential is due to activation of $G_{K(Ca)}$ and the other K-currents (Brown *et al.*, 1982; Galvan & Sedlmeir, 1984;

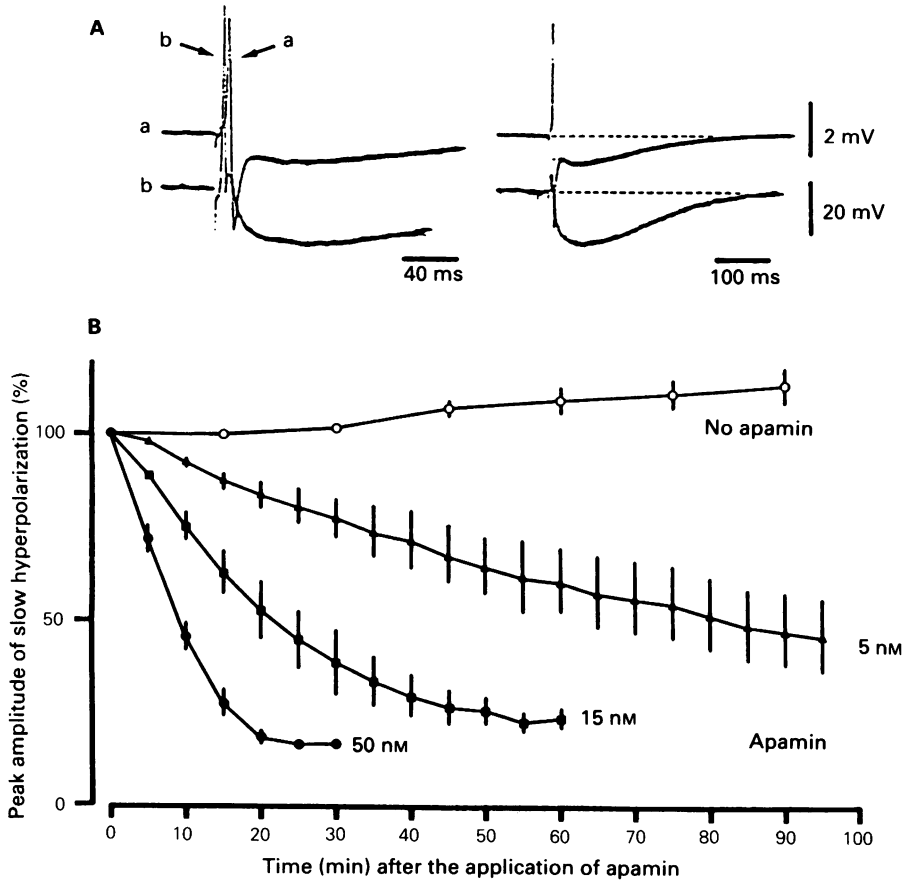


**Figure 3** Effects of apamin on potentials recorded extracellularly. (A) Action potentials and after hyperpolarization (AH) recorded simultaneously by suction electrode (a) and intracellular microelectrode (b). The preganglionic nerve was stimulated with a rectangular pulse supramaximal in voltage and 0.2 ms in duration. Note that right hand panel is shown at slower sweep than left hand one. The action potentials have been slightly retouched. (B) Time course of the inhibitory effect of apamin on the peak of the slow hyperpolarization recorded extracellularly. Extracellular $Ca^{2+}$ was increased to 4 mM to allow better discrimination of slow and fast components. Symbols indicate in the absence (○) and in the presence of 50 nM (●), 15 nM (■), 5 nM apamin (▲). Vertical bars indicate s.e.mean, $n = 4$.

Belluzzi *et al.*, 1985). In the present studies, apamin affected neither duration nor maximum rate of fall of the Na spike, but prolonged the Ca spike. The fact that apamin prolongs the Ca spike rules out the possibility that the effect of apamin is voltage-dependent, i.e. apamin exerts its effect only around the resting potential. This may be explained by the slow onset of $I_{K(Ca)}$, allowing it to contribute to the repolarization of Ca spike which has a much slower time course than the Na spike. It might be that there are two components of $G_{K(Ca)}$, as shown in the bullfrog sympathetic ganglion (Pennefather *et al.*, 1985). If this is so, it is likely that the apamin-sensitive component of $G_{K(Ca)}$, slowly activates to contribute the AH and the repolarization of Ca-spike and the apamin-resistant component activates rapidly and repolarizes the Na-spike . Since in the presence of 10 mM TEA, the Ca-spike was followed by a large AH, the apamin-sensitive component may be rather resistant to TEA, as shown in other tissues (Romey *et al.*, 1984; Pennefather *et al.*, 1985).

Further, the finding that apamin decreases accommodation and increases repetitive firing during current injection suggests that the AH or the activation of the $G_{K(Ca)}$ has physiological significance in regulating firing. Brown *et al.* (1982) have reported that muscarine, which blocks M-current, decreases accommodation. In line with the observation of Pennefather *et al.* (1985), it was unlikely that apamin

blocked M-current, since apamin affected neither the resting potential, input resistance nor shape of electrotonic potentials.

Since ganglionic transmission was unaffected, it is clear that apamin did not block nicotinic receptors. Almost all of the slow component of AH following the

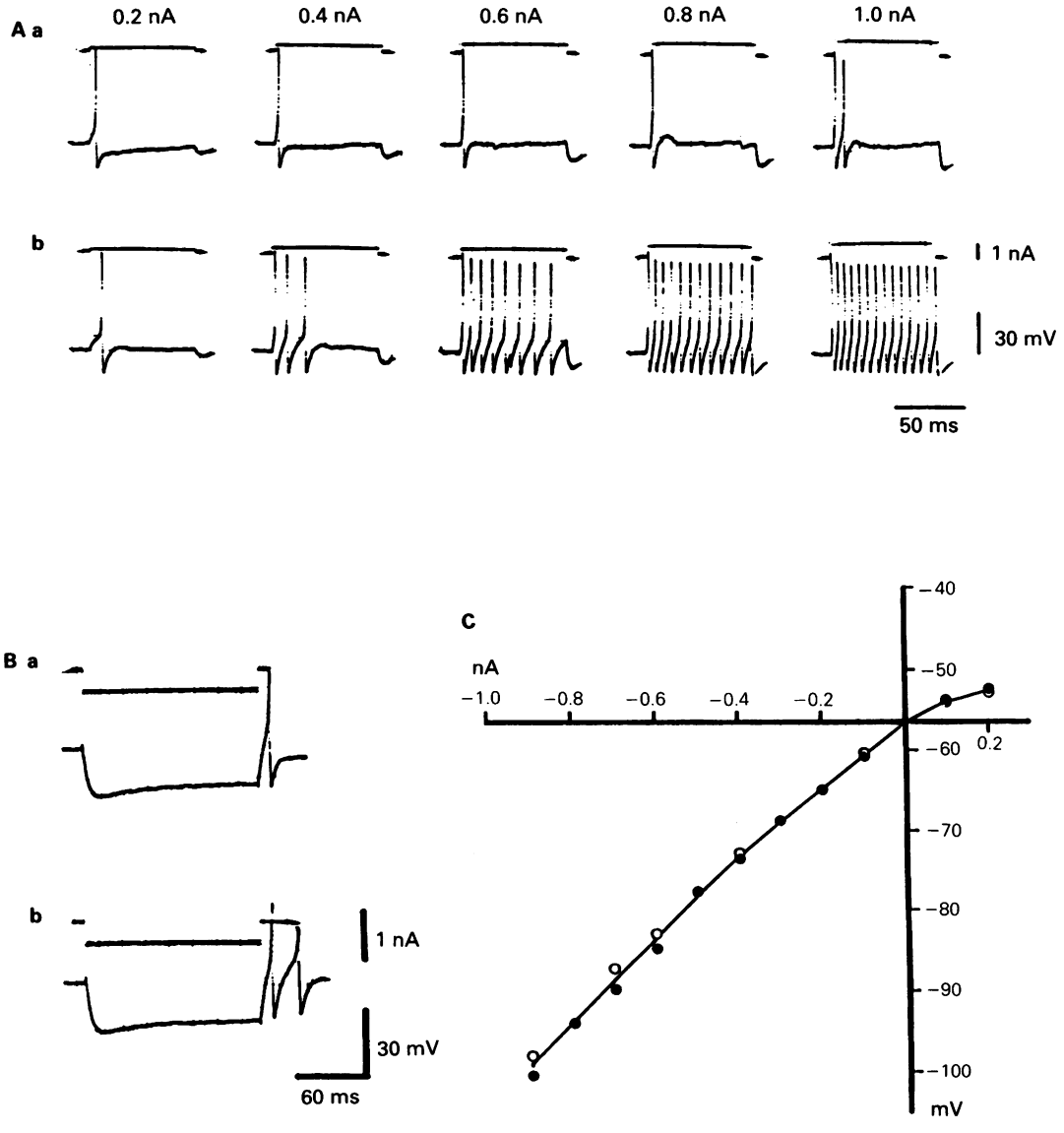


**Figure 4** Effects of apamin on responses to depolarizing (A) and hyperpolarizing (B) current pulses and on the I-V relationship (C). In (A) and (B) (a) controls; (b) in the presence of 50 nM apamin. In (B) the peak of anodal-break spikes is not shown. In (C) symbols indicate control (●) and in the presence of 50 nM apamin (○). The membrane potentials were measured at the end of a pulse of 150 ms in duration. Similar records were obtained from more than 10 cells.

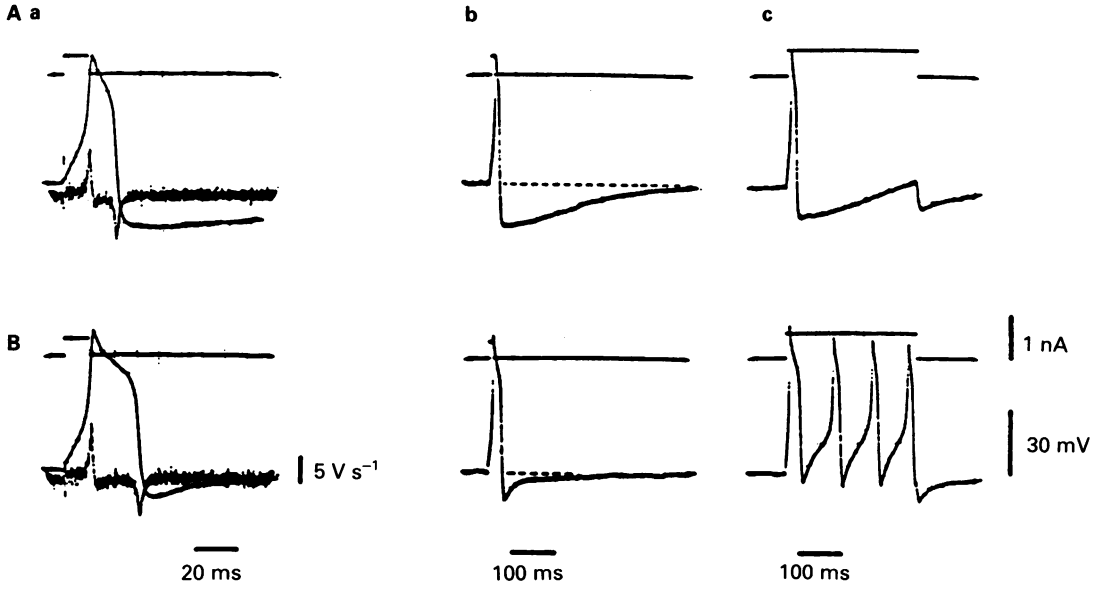


**Figure 5** Effects of apamin on Ca spike and AH in the presence of TTX 1 μM, TEA 10 mM and $Ca^{2+}$ 4 mM. Applied currents are 10 ms (a and b) and 300 ms (c). (A) control; (B) 50 nM apamin. Similar records were obtained in 6 different cells.

orthodromic action potential consists of the apamin-sensitive $G_{K(Ca)}$, possibly because the apamin-resistant fast component of the AH is masked by the e.p.s.p. Transmission failure was often observed during the AH, if the stimulus was submaximal, but no failure occurred when the AH was blocked by apamin. Thus the AH may have physiological significance in regulating synaptic transmission by modulating the excitability of post-ganglionic neurones, such that the effect of a few weak inputs following a spike are suppressed. There is also the possibility that if $G_{K(Ca)}$ exists in the presynaptic terminal (Mallart, 1984), it may modulate release of acetylcholine during trains of impulses.

The apamin-induced blockade was not use-dependent, i.e. it was not necessary for the $G_{K(Ca)}$ channels to be open for blockade to occur. Therefore, specific receptors for apamin may exist. The time to maximum blockade of $G_{K(Ca)}$ was strongly dose-dependent. In superficial cells, the blockade was rapid in onset but poorly reversible. These results may indicate that apamin penetrates poorly into the interior of the ganglionic tissue; it may also be decomposed during diffusion to effective sites. The rate of dissociation from its binding sites has been reported to be very slow (Hugues *et al.*, 1982a). The $ID_{50}$ for inhibition of the AH was estimated as 15 nM, 20 min after the application of apamin to the whole ganglion. If diffusion is allowed for, the $ID_{50}$ at steady state is possibly less than nanomolar, which is as low as the value estimated from K-loss by hepatocytes (1 nM; Cook *et al.*, 1983).

Cook & Haylett (1985) have reported that $^{125}$I-apamin binding in hepatocytes is inhibited by (+)-tubocurarine and hexamethonium; it is likely that these blockers and apamin bind to identical sites to block $G_{K(Ca)}$. Hexamethonium inhibits AH in the sympathetic neurone of rat and bullfrog (Kawai *et al.*, 1985) and similar effects of (+)-tubocurarine have been observed in bullfrog (Nohmi & Kuba, 1984) and rat (Kawai & Watanabe, unpublished). These observations may indicate that apamin-sensitive channels in these preparations are pharmacologically very similar, probably at least in their apamin-binding sites.

In conclusion, in rat sympathetic neurones apamin potently suppresses the slow component of AH by directly blocking $G_{K(Ca)}$, resulting in an increase in excitability of the cells. Using apamin, rat sympathetic neurones may be a useful preparation for investigating the $G_{K(Ca)}$ system, its kinetic properties and physiological functions.

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