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# Modulation of Membrane $K^+$ Conductance in T-Lymphocytes by Substance P via a GTP-Binding Protein

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**Summary.** Modulation of the voltage-gated $K^+$ conductance in T-lymphocytes by substance P was examined. Whole-cell recordings from Jurkat E6-1 human T-lymphocytes revealed two components of substance P action on the outward $K^+$ current: (i) dose- and time-dependent reduction of current peak amplitude; and (ii) acceleration of the current inactivation rate. This action was blocked by substituting $Cs^+$ for $K^+$ in the recording pipette and by the substance P antagonist, [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P. As indicated by conductance-voltage relationship, the reduction in current peak amplitude as a result of substance P application was not due to a shift of the voltage dependence of the channel. Raising intracellular free calcium concentration from 2 to 200 nM reversed the reduction, induced by substance P, in current peak amplitude and disclosed an apparent desensitization towards the neuropeptide action. The treatment, however, did not reverse substance P-induced acceleration of the rate of current decay. Intracellular administration of hydrolysis-resistant guanosine triphosphate (to persistently activate GTP-binding protein) and guanosine diphosphate (to competitively inhibit GTP—binding proteins) analogues mimicked and inhibited substance P-induced reduction of $K^+$ conductance, respectively. The data demonstrate a modulation of T-lymphocyte $K^+$ channels by substance P and substantiate a possible role for GTP-binding proteins in this modulation.

**Key Words** T-lymphocyte · substance P · $K^+$ current · desensitization · GTP-binding protein · substance P antagonist

## Introduction

The sensory neuropeptide substance P (SP) mediates inflammation [15] and stimulates T-lymphocyte proliferation [21] and lymphokine secretion [20]. Coupled with the expression of SP receptors on T-lymphocytes [22], the findings support a role for SP in immunoregulation. The precise molecular mechanism of SP action on the lymphocyte has been undefined, however. It is known that a primary action of SP on neurons is to reduce membrane voltage-dependent $K^+$ conductances including the M-current [1] and inwardly rectifying current [29]. The presence of a voltage-gated, outwardly rectifying $K^+$ conductance in T-lymphocytes has been demonstrated [6, 8, 11, 17, 18, 26]. Roles for the $K^+$ channel in T-lymphocytes in maintenance of resting membrane potential, mitogenesis, and volume regulation [3, 7, 9] have been postulated. We questioned whether SP modulates the voltage-gated $K^+$ conductance in T-lymphocytes in a fashion similar to neurons and thereby influences T-lymphocyte function.

The aim of the present study was (i) to electrophysiologically characterize the modulation of $K^+$ currents in T-lymphocytes by SP and (ii) to test for possible involvement of second messengers in the modulatory process. We used the whole-cell recording variation of the patch-clamp technique [12] to assess the effect of SP on macroscopic $K^+$ current in Jurkat T-lymphocytes. After activation by depolarization, the current was examined for its amplitude and kinetics prior to and following bath application of SP. As intracellular free calcium concentration ($[Ca^{2+}]_i$) may affect $K^+$ conductance [2], the action of SP on the conductance was investigated in low and high $[Ca^{2+}]_i$. Finally, possible involvement of GTP-binding proteins was investigated by determining the effect of intracellular administration of hydrolysis-resistant guanosine di- and triphosphate analogues on SP-modulated $K^+$ current.

Our results indicate that SP reduces $K^+$ conductance in low $[Ca^{2+}]_i$ ($2 \times 10^{-9}$ M) by decreasing current peak amplitude and accelerating the rate at which the current inactivates. The data also support a possible role for GTP-binding proteins in the modulation of T-lymphocyte $K^+$ channels by SP. A preliminary report of this work has been presented in abstract form [27].

## Materials and Methods

### Cell Culture

All experiments were performed on cells of the malignant T-lymphocyte cell line Jurkat E6-1, which were maintained in medium RPMI-1640 (with HEPES), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 50 $\mu g\ ml^{-1}$ gentamycin, 2 mM L-glutamine (Gibco) at 37°C in humidified 5% $CO_2$ atmosphere. Before recording, the cells were washed twice and resuspended in the bathing solution listed below. 3-ml aliquots of cell suspension were placed in 35-mm dishes for recordings.

### Solutions (Composition in mmol/Liter)

The bathing solution has the following composition: 154 NaCl, 5.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES at pH 7.4 (adjusted with NaOH). KCl was substituted, on equimolar basis, for NaCl in the bathing solution to obtain a solution with [KCl] equal to the concentration in the pipette (internal) solution. The internal solution was composed of 150 KCl (or CsCl to block $K^+$ current), 1 $CaCl_2$, 11 EGTA (resulting in approximately 2 nM free $[Ca^{2+}]_i$, *see* ref. [2]), 2 $MgCl_2$ and 10 HEPES at pH 7.2 (adjusted by KOH). To have an internal solution with higher free $[Ca^{2+}]$ only 1.1 EGTA was included (resulting in approximately 200 nM free $[Ca^{2+}]_i$). All solutions were filtered through 0.2 $\mu$m millipore filters just before use.

### Other Chemicals

The neuropeptide, SP, and its antagonist, [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-SP were purchased from Peninsula Laboratories. SP was directly aliquoted into a fresh bath for each assay. To block SP action, the antagonist and SP were applied concurrently to the bath. Guanosine-5′-O-(2-thiodiphosphate) (GDP-$\beta$-S), trilithium salt and guanosine-5′-O-(3-thiotriphosphate) (GTP-$\gamma$-S), tetralithium salt were purchased from Boehringer Mannheim Biochemicals. Guanosine derivatives were included in the pipette for 5-min dialysis before recording to achieve equilibrium with cell cytoplasm.

### Recording

The whole-cell configuration of the patch-clamp technique [12] was used throughout to voltage clamp single cells of nearly equal sizes. Series resistance compensation to decrease access resistance was usually unnecessary, since the pipette input resistances were low (3–6 MΩ) compared to cellular input resistance (5–10 GΩ). Residual liquid junction potentials were compensated electronically before establishing contact with the cell. Pipette-membrane seal resistance was >10 GΩ. After formation of giga-seal, strong negative pressure was applied in order to obtain whole-cell clamp, which was indicated by a sudden increase in the capacitative surge and the membrane noise or by abrupt negative transition of recording potential in current clamp. All voltage-clamp experiments were made at room temperature (20–22°C).

Recording was done using Axopatch-1B (Axon Instruments) patch-clamp amplifier with a 10 GΩ feedback resistor and active lowpass filtering at 5 kHz. Data acquisition and storage of current records during the execution of voltage-clamp protocol were achieved by implementing pClamp Clampex program (Axon Instruments) through a microcomputer (IBM PC-AT) interfacing by an analog-to-digital converter (a Labmaster board) with the recording arrangements. Measurements and fitting analysis is done by means of Clampfit program (Axon Instruments).

Families of voltage-dependent $K^+$ currents were evoked in Jurkat cells by depolarizing pulses delivered in 10-mV steps, from −30 to +50 mV, at a frequency of 0.1 Hz and duration of 50 msec to allow complete recovery from inactivation. Throughout, the step commands were given from a holding potential of −70 mV. The leakage current was estimated from 10-mV depolarizing pulses. Membrane $K^+$ conductance was calculated as follows: The derived value, after subtraction of the scaled leak from peak current induced by each potential step, was then divided by the potential minus the reversal potential whose calculated value equaled −84 mV according to Nernst equation. Data were expressed as the mean ± SEM and analyzed by Student's $t$ test and the least significant difference to compare between means.

## Results

### Substance P Action on Whole-Cell $K^+$ Currents

We used the whole-cell recording configuration of the patch-clamp technique to assess the effect of SP on macroscopic current in Jurkat T-lymphocytes. Figure 1 (upper traces) shows typical $K^+$ currents recorded under control conditions. Voltage steps from a holding potential of −70 mV to potentials of more than −30 mV produced outward $K^+$ currents with kinetics and amplitudes similar to those previously described [6, 8, 11, 17]. Confirmation that the depolarization-induced outward currents were carried by $K^+$ ions was provided by the following: (i) If the extracellular $K^+$ concentration was raised to the concentration within the pipette, inward $K^+$ currents were observed below a well-defined reversal potential of near zero mV, and (ii) substituting $Cs^{2+}$ ions for $K^+$ ions in the pipette abolished the outward current.

Since the time of assessing SP action was 7.5 min, it was crucial to monitor the $K^+$ current over this time period in control conditions. Under these conditions, 36 of 45 cells produced nearly similar $K^+$ current responses, with respect to peak amplitude and rate of current inactivation, over the more than 8-min recording period. In nine cells a continued decremental response in the $K^+$ current ensued over the first minute of recording, causing a loss of >40% of the maximum conductance within 5 min. Therefore, cells were observed for about 5 min prior to experimentation with SP; cells with rundown responses were discarded, and cells that repeatedly showed stable response were considered to be stable controls.

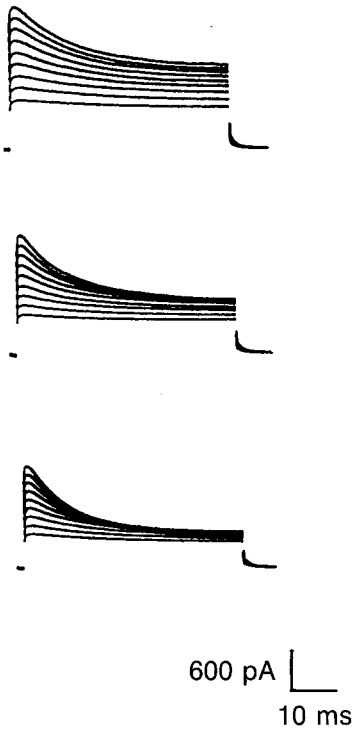


**Fig. 1.** Action of SP on whole-cell outward $K^+$ current elicited in T-lymphocytes. A family of voltage-dependent $K^+$ currents was evoked in Jurkat cells in the whole-cell configuration by depolarizing pulses delivered in 10-mV steps, from −30 to +50 mV, at a frequency of 0.1 Hz and duration of 50 msec to allow complete recovery from inactivation. Voltage steps were applied from a holding potential of −70 mV. Records of this particular cell have no subtraction of linear leakage currents. The upper trace is the control response recorded before administering SP. SP (1 μM) was applied to the recording bath medium. Middle and lower traces were recorded 1 and 7.5 min after the treatment with SP

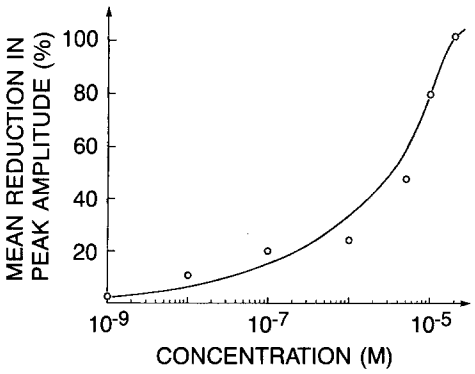


**Fig. 2.** Dose-response relationship (a semilog plot) for SP action on the $K^+$ current peak amplitude in T-lymphocytes. Measurements were taken from cells of nearly equal sizes and from current responses produced by a 120-mV depolarizing step from a holding potential of −70 mV. Currents were recorded before and 7.5 min after bath application of SP. Each point indicates the mean value among five tested cells. Correction for the rundown current (<6%) was taken into account

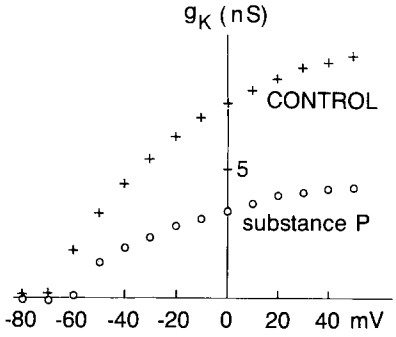


**Fig. 3.** Voltage-dependence of T-lymphocyte $K^+$ conductance. Corresponding peak chord conductance to voltage relations were plotted (same cell in Fig. 1) with a maximum conductance of 9.5 and 5.3 nS in the control (+) and after SP (○) application, respectively. Currents were measured at their peak amplitudes and their values were derived after subtraction of a linear component of leak current. *See* Materials and Methods for the calculation of the data points

Having achieved stable whole-cell recording, administration of SP resulted in reduction in $K^+$ current peak amplitude that was both time- (Fig. 1) and dose-dependent (Fig. 2). While there was cell-to-cell variability in terms of $K^+$ current peak amplitude, which may be attributed to differences in cell size or channel density, almost all cells responded to SP with similar sensitivity. After correction for the anticipated rundown (<6%) in control response, a partial dose-response curve was constructed (Fig. 2). The estimated $EC_{50}$ value of SP was 2 μM, and the range of tested SP concentrations producing detectable responses was from 1 nM to 20 μM. The overall SP-induced decrease in the $K^+$ current peak amplitude was not due to displacement of the voltage dependence of the channel, as can be shown by conductance-voltage plots (Fig. 3). SP action on $K^+$ conductance was specific, since SP-sensitive current showed reversal of polarity at a membrane potential near to the equilibrium potential of $K^+$ ($E_k$), and since replacing $K^+$ ions with $Cs^+$ ions in the pipette abolished the SP effect and revealed no SP-induced inward current (*not shown*).

In addition to reducing $K^+$ peak conductance, SP at several concentrations accelerated the current inactivation rate, as indicated by the smaller time constant in 14 out of 19 cells (Fig. 4). The estimated reduction of the time constant as a result of the application of 5 μM SP in five cells was 46.3 ± SEM 6.8%.

Partial pharmacologic blockade of SP action was possible by one of SP analogues, [D-Arg$^1$, D-

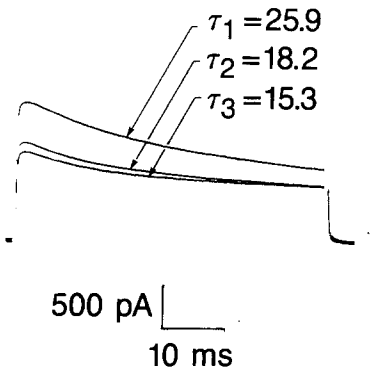


**Fig. 4.** Effect of SP on the rate at which the whole-cell outward $K^+$ current inactivates. Three traces representing the current induced at 120-mV voltage step in one cell, from a holding potential of −70 mV, before (trace *1*), and 1 min (trace *2*) and 3 min (trace *3*) after SP (5 $\mu$M) administration. The corresponding values (in msec) of the time constant were obtained by fitting the trace with a single exponential decay (linear regression >0.99)

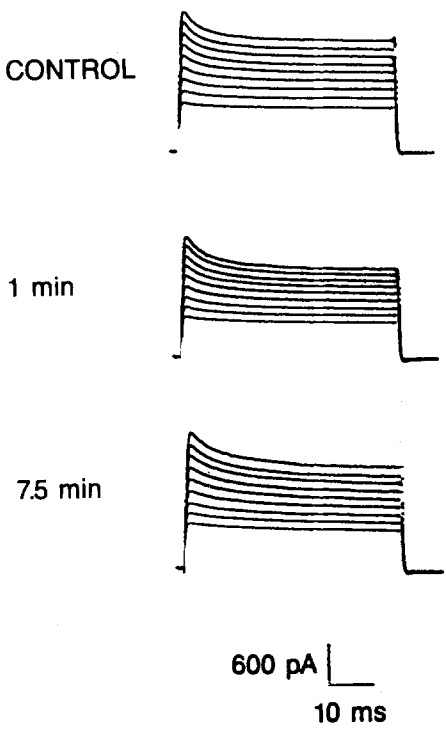


**Fig. 5.** Effect of high (200 nM) $[Ca^{2+}]_i$ on the action of SP on whole-cell outward $K^+$ current elicited in T-lymphocytes. A family of voltage-dependent $K^+$ currents was evoked in a Jurkat cell with a protocol identical to that listed in Fig. 1. The pipette filling solution contained 200 nM $Ca^{2+}$ (adjusted by 1.1 mM of EGTA as indicated in Materials and Methods). The upper trace represents a control response before the administration of SP. The middle and lower traces were recorded from the same cell 1 and 7.5 min after treatment with 5 $\mu$M SP, respectively

Phe[5], D-Trp[7,9], Leu[11]]-SP. The analogue has been found to be a competitive antagonist in the guinea-pig ileum [30]. Applied concurrently with SP, the antagonist at 50 $\mu$M blocked SP (10 nM)-induced reduction in peak amplitude by 94.3 ± SEM 1.3% (for five cells). The antagonist by itself at the same concentration did not affect the characteristics of the control responses ($n$ = 8).

### Effect of Internal Calcium Concentration on SP Action

It has been reported that elevation of $[Ca^{2+}]_i$ to 10 nM or more reduces $K^+$ conductance in human T-lymphocytes [2]. Since we used a pipette solution whose free $[Ca^{2+}]$ is kept at 2 nM (*see* Materials and Methods), the suppression of $K^+$ conductance by SP did not appear to be due to a mechanism that involves raising $[Ca^{2+}]_i$. To further investigate this, we raised the free $[Ca^{2+}]$ in the pipette solution to 200 nM in contrast to 2 nM $[Ca^{2+}]$. In higher $[Ca^{2+}]_i$, the initial SP-induced decrease in current peak amplitude reversed to near control value over the 7.5-min recording period (Fig. 5). We drew two conclusions from this result. First, it is unlikely that $Ca^{2+}$ is the secondary messenger in the modulation of $K^+$ current by SP, since SP, at higher $[Ca^{2+}]_i$, appears to be less potent at reducing $K^+$ current peak amplitude at all tested concentrations (Table). Second, the recovery from SP action at the higher $[Ca^{2+}]_i$ implied a possible desensitization, which is both time- and $Ca^{2+}$-dependent (Fig. 6). It should be noted that in contrast to the SP effect on $K^+$ current peak amplitude, the SP effect on the rate of current inactivation was irreversible in 19 of 28 cells, even at higher $[Ca^{2+}]_i$.

### Possible Involvement of a GTP-Binding Protein in $K^+$ Modulation

To further characterize the mechanism by which SP modulates $K^+$ channels in T-lymphocytes, we tested for possible GTP-binding protein involvement in the modulatory process. There is precedence for this. Pertussis toxin-insensitive GTP-binding proteins have been reported to mediate SP-induced inhibition of inwardly rectifying $K^+$ channels in brain neurons [19]. To test whether G-proteins may mediate the SP action on lymphocyte $K^+$ channels, we used the nonhydrolyzable GTP and GDP analogues which irreversibly activate and competitively inhibit, respectively, the GTP-binding proteins [10, 25].

Figure 7 shows an experiment in which the intrapipette solution contained 50 $\mu$M GTP-$\gamma$-S. The

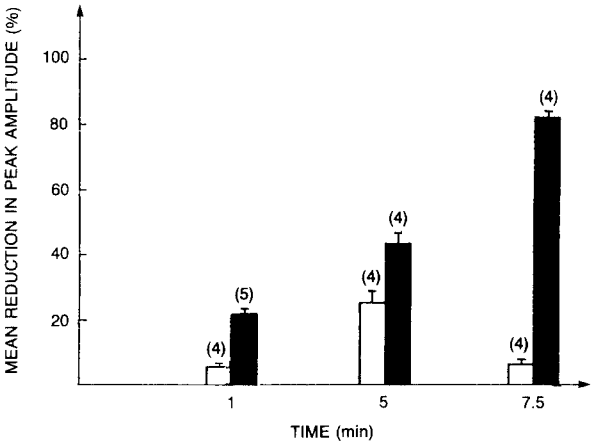


**Fig. 6.** Time-dependent action of SP on the $K^+$ current peak amplitude in low (2 nM) and high (200 nM) $[Ca^{2+}]_i$. Measurements were taken only from current responses produced by 120-mV depolarizing step from a holding potential of −70 mV. The bars represent the mean percent reduction (in relation to control value) in peak amplitude, induced by 5 μM SP, as a function of time, and low (filled bars) and high (open bars) $[Ca^{2+}]_i$ conditions. The open bars (from cells similar to cell shown in Fig. 6.) show time-dependent current decrease, over the initial 5 min, which is reversed 2.5 min later. The filled bars (from cell similar to cell shown in Fig. 1.) show continuous time-dependent action of SP. The error bar is SEM and numbers in parentheses represent the number of tested cells

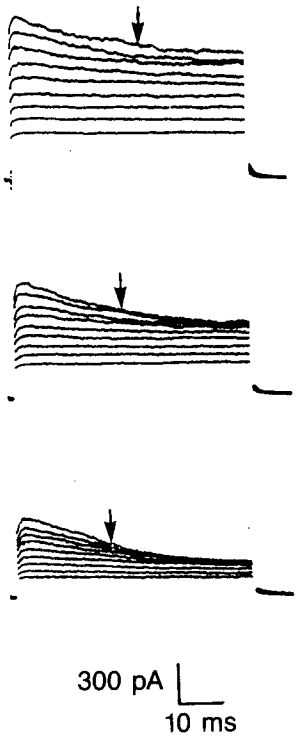


**Fig. 7.** Whole-cell current records from a Jurkat T-lymphocyte dialyzed for 5 min with 50 μM GTP-γ-S (tetralithium salt, Boehringer Mannheim) by inclusion of the analogue in the pipette solution. The protocol of voltage clamp was the same as in Fig. 1. Upper, middle and bottom traces were recorded 1, 3 and 5 min after dialysis with GTP-γ-S, respectively. Arrows indicate half decay times

**Table.** Potency of SP in two different $[Ca^{2+}]_i$ conditions[a]

| Mean reduction in peak amplitude of the whole-cell $K^+$ current (%) | | |
|---|---|---|
| Substance P conc. (μM) | EGTA (1.1 mM)[b] | EGTA (11 mM) |
| 0.1 | 5.0 ± 1.5 (5)[c] | 15.5 ± 2.5 (5)[d] |
| 1.0 | 8.5 ± 1.8 (4) | 19.4 ± 3.2 (5)[d] |
| 5.0 | 15.9 ± 1.9 (7) | 21.3 ± 1.1 (5)[d] |
| 10.0 | 33.5 ± 1.8 (5) | 44.7 ± 3.4 (4)[d] |

[a] Measurements were taken 3 min after SP administration from responses elicited by a 120-mV pulse from a holding potential of −70 mV.

[b] Pipette solution was adjusted for free $[Ca^{2+}]_i$, using 1.1 and 11 mM EGTA, so that a high (200 nM) and a low (2 nM) $[Ca^{2+}]_i$ can be produced, respectively.

[c] Mean ± SEM (number of cells).

[d] $P < 0.01$ (using the least significant difference as a statistical test to compare between means).

intracellular application of GTP-γ-S alone produced a time-dependent decrease in $K^+$ current peak amplitude and time constant of inactivation. The GTP-γ-S-induced reduction in current peak amplitude, calculated to be 30 ± 2.2% ($n = 8$) at 1 min after the dialysis, was equivalent to that induced by 7.5 μM SP after 1 min of incubation with the neuropeptide. Administering SP (5 nM) while recording with a dialysing pipette containing GTP-γ-S (50 μM) produced an enhanced decrease in current amplitude (averaging 52% for three cells). We also tested the effect of a GDP derivative, GDP-β-S, which prevents the reaction of GTP-binding proteins with GTP [10]. The intracellular application of GDP-β-S (100–500 μM) blocked the SP (5 μM)-induced decrease in $K^+$ current peak amplitude in a dose-dependent manner (Fig. 8). Together, the actions of GTP-γ-S in mimicking SP action and GDP-β-S in inhibiting SP action strongly support a possible role for GTP-binding proteins in coupling the SP signal to the $K^+$ channel.

## Discussion

We studied the modulation of the membrane $K^+$ conductance by SP in Jurkat T-lymphocytes. We established that the modified conductance was a $K^+$ conductance by the following observations: (i) The

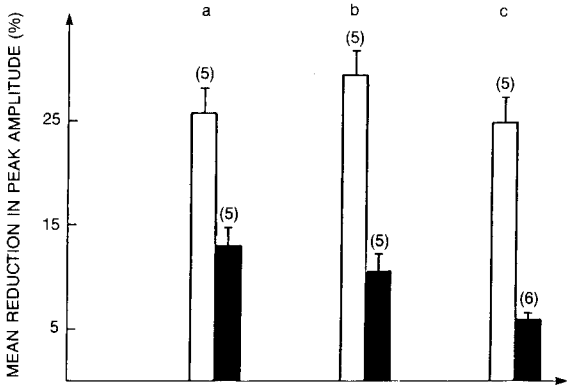


**Fig. 8.** Blockade of T-lymphocyte response to SP by GDP-β-S. Cells were dialyzed for 5 min with 100 (*a*), 250 (*b*), and 500 (*c*) μM GDP-β-S (trilithium salt, Boehringer Mannheim). Measurements were taken from $K^+$ current evoked from a holding potential of −70 mV by a 120-mV depolarizing pulse. The mean decrease in $K^+$ current in response to SP (5 μM), applied for 1 min, is indicated for untreated control cells (open bars) and cells dialyzed with GDP-β-S (filled bars). The responses to SP obtained from cells treated with GDP-β-S were compared with responses recorded from untreated cells in the same culture dish. Error bars indicate SEM and number of cells is shown in parentheses. GDP-β-S did not directly inhibit $K^+$ current, nor did it accelerate rundown of the current

fall in membrane conductance induced by SP was seen more clearly if the membrane was depolarized, implying that an outwardly rectifying conductance is affected. (ii) This conductance depended upon external $K^+$ concentration as well as on voltage, and the SP-sensitive current reversed at a membrane potential similar to $E_k$. (iii) The SP-sensitive conductance was impermeable to cesium. The fall in voltage-gated $K^+$ conductance by SP is reminiscent of the neuropeptide action on sympathetic [1] and brain neurons [19, 29].

The effect of SP on membrane $K^+$ conductance seems to be $Ca^{2+}$-dependent. A rise in $[Ca^{2+}]_i$ made the T-lymphocyte $K^+$ conductance less sensitive to SP and caused apparent desensitization. There are other examples for SP-induced desensitization in various systems [14, 28] and for the involvement of $[Ca^{2+}]_i$ in the desensitization towards hormonal action [24].

It is known that GTP-binding proteins may directly interact with ion channels or may couple with other second messenger-generating systems to achieve a modulatory effect. In this case, experimental findings argue for a direct role of GTP-binding proteins as second messengers. Involvement of adenylyl cyclase seems unlikely, since agents known to increase intracellular cAMP fail to inhibit lymphocyte $K^+$ conductances [13]. In addition, an adenylate cyclase-based response for SP has not been convincingly demonstrated [19]. Similarly, involvement of polyphosphoinositide turnover seems unlikely, since phorbol esters enhance the $K^+$ conductance in T-lymphocyte [8] and rises in $[Ca^{2+}]_i$ reverses SP-induced inhibition of $K^+$ conductance (indicated by the present results). These preliminary observations argue against two GTP-binding protein-linked second messenger systems in the SP signal transduction process. Alternatively, GTP-proteins may directly modulate $K^+$ channels, as has been documented in nonlymphoid cells [5, 23] and possibly B-lymphocytes [4]. A pertussis toxin-insensitive GTP-binding protein has been reported to mediate SP-induced inhibition of inward rectifying $K^+$ channels in brain neurons [19]. Our results indicating the effect of GTP-γ-S in mimicking SP action and GDP-β-S in inhibiting SP action strongly support a possible role for a GTP-binding protein in directly coupling the SP signal to the $K^+$ channel. This may be another example of a direct role of a GTP-binding protein in modulating $K^+$ channels; however, further investigation is needed to document this interpretation.

The precise significance of reducing the $K^+$ conductance in T-lymphocytes remains to be determined. These results, however, combined with the presence of high affinity SP receptors on lymphocytes [22], and the effects of SP on lymphocyte proliferation [21], lymphokine secretion [20], and cytokine production [16] strongly support a role for SP in the neural regulation of immune function.

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